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Phosphatidylinositol Cycle and Bipolar Disorder: Investigation of Intracellular Calcium

by

Michele Leslie Ulrich ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of *Masters of Science*

Department of Psychiatry

Edmonton, Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Phosphatidylinositol Cycle and Bipolar Disorder: Investigation of Intracellular Calcium* submitted by *Michele Leslie Ulrich* in partial fulfillment of the requirements for the degree of *Master of Science*

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Date: Dec. 20, 2000

Dedication

This thesis is dedicated to my dad, Gordon, my brothers, Geoffrey and Gregory, and in remembrance of my mother, Donna, all of whom have been greatly supportive of, and interested in my academic endeavors.

Abstract

There is extensive evidence implicating the phosphatidylinositol (PI-) cycle in the etiology and pathophysiology of bipolar disorder and the therapeutic mechanisms of mood stabilizers. Enhanced increases in intracellular calcium ions (Ca^{2+}) induced by agonists that activate the PI-cycle has been found in peripheral blood cells of bipolar manic and depressed patients. A fluorometric assay to measure platelet Ca^{2+} concentrations from healthy male subjects using Fura-2 is described. *In vivo* or *in vitro* dextroamphetamine (a model of mania) had no effect on the increase in platelet Ca^{2+} induced by thrombin- or 5-HT, while *in vitro* dextroamphetamine increased basal Ca^{2+} concentrations. Mood stabilizers, lithium, sodium valproate and carbamazepine, incubated with platelets *in vitro* increased basal Ca^{2+} concentrations, while carbamazepine enhanced the increase in Ca^{2+} induced by thrombin- and 5-HT. The increase in platelet intracellular Ca^{2+} produced by mood stabilizers may be a result of a transient build-up of IP_3 following acute inhibition of the PI-cycle.

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List of Abbreviations

ACD	acid-citric acid-dextrose
Ach	acetylcholine
AD	antidepressant
AH	antihypertensive
AlF_4	aluminum fluoride
AM	acetoxymethyl ester
Amph	dextroamphetamine
AMPH	dextroamphetamine
ADP	adenosine diphosphate
AP	antipsychotic
ATP	adenosine triphosphate
BAPTA	1,2-bis (o-aminophenoxy) ethane-N,N,-N′,N′-tetraacetic acid
BP	bipolar
C	control
Ca^{2+}	calcium ion
$[Ca^{2+}]$	concentration of calcium ions
CBZ	carbamazepine
CCD	citrate-citric acid-dextrose
CCK	cholecystekinin
CDP-DG	cytidine diphosphate diacylglycerol
CMP-PA	cytidine monophosphate phosphatidate
cPKC	Ca^{2+}-dependent protein kinase C
DA	dopamine
DAG	sn-(1,2)-diacylglycerol
E	epinephrine
ECG	electrocardiogram
ECS	electroconvulsive shock
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ER	endoplasmic reticulum
F	fluorescent intensity, fluoresence
F_{min}	fluorescent intensity in the presence of minimal $[Ca^{2+}]$
F_{max}	fluorescent intensity in the presence of maximal $[Ca^{2+}]$
fMLP	formylmethionylleucylphenylalanine
Fura-2AM	acetoxymethylester form of Fura-2
GTP	guanine triphosphate
$GTP\gamma S$	guananine triphosphate-γS
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
5-HT	5-hydroxytryptamine (serotonin)

^{1}H	proton nucleus
HC	healthy control
I-1-P	inositol-1-phospohates
ip	intraperitoneal injection
IP	inositol phosphate(s)
I(x)P	inositol monophosphates
$I(x,x,)P_2$	inositol bisphosphates
$I(x,x,x)P_3$	inositol trisphosphates
IP_3	inositol-(1,4,5)- trisphosphate
IP_2	inositol bisphosphate(s)
IP_4	inositol-(1,3,4,5)P_4
IP_3R	inositol trisphosphate I(1,4,5)P_3 receptor
$I(x,x,x,x)P_4$	inositol tetrakisphosphates
IMPase	inositol monophosphatase
IPPase	inositol polyphosphate-1-phosphatase
$G_{q/11}$	G-protein subtype
GTPγS	guanine triphosphate-gamma-sulphate
K	Kelvin
K_a	association constant
K_d	dissociation constant
Li	lithium chloride
M	molar
MAO	monoamine oxidase
MAO_B	monoamine oxidase B
MARCKS	myristolated alanine rich C kinase substrate
meq/kg	milliequivalents per kilogram of animal weight
MNL	mononuclear lymphocyte
µM	micromolar
mM	millimolar
MRS	magnetic resonance spectroscopy
NaF	sodium fluoride
NAA	N-acetylaspartic acid
NE	norepinephrine
NSAIDS	non-steroidal anti-inflammatory drugs
ng	nanograms
nL	nanoliters
nm	nanometers
nM	nanomolar
nPKC	Ca^{2+}-independent protein kinase C
^{31}P	phosphorus nucleus
PA	phosphatidic acid
PAF	platelet activating factor
PCr	phosphocreatine
PDBu	phorbol 12,13-dibutyrate
PDE	phosphodiester
PG	prostaglandins

PGI$_2$	prostaglandin I$_2$, prostacyclin
PHG	phytohemagluttinin
Pi	inorganic phosphate
PI	phosphatidylinositol
PIP	phosphatidylinositol phosphate
PIP$_2$	phosphatidylinositol-(4,5)- bisphosphate
PKC	protein kinase C
PLAC	placebo
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PME	phosphomonester(s)
PPP	platelet-poor plasma
PRP	platelet-rich plasma
R	ratio of fluorescent signals
R$_{min}$	ratio of fluorescent signals in the presence of minimal Ca^{2+}
R$_{max}$	ratio of fluorescent signals in the presence of maximal Ca^{2+}
RM-ANOVA	repeated measures analysis of variance
sc	subcutaneous injection
SDS	sodium dodecyl sulphate, lauryl sulphate
SEM	standard error of the mean
TPEN	N,N,N′,N□-tetrakis (2-pyridylmethyl) ethylenediamine
TRIS	2-(hydroxymethyl) aminomethane
UP	unipolar
UV	ultraviolet
VPA	sodium valproate

CHAPTER 1 – BACKGROUND LITERATURE REVIEW

1.1 Introduction

Bipolar (manic-depressive) disorder is a psychiatric condition present in approximately 1% of the general population and characterized by cycles between elated and depressed mood states (American Psychiatric Association 1994). The personal and professional lives, as well as the mental and physical well being, of the patients can be seriously jeopardized as a result of the high risk-taking activity and suicidal tendencies which can occur. The significantly increased prevalence rates within families and the high rates of therapeutic response to pharmacological agents strongly suggest a biological basis. However, the etiology and neuronal pathophysiology of bipolar disorder and the therapeutic mechanisms of action of medications remain unknown. Pharmacotherapy with mood stabilizers remains the most effective treatment for both acute and long-term symptom reduction in bipolar patients. For many years the standard treatment of bipolar disorder has been lithium carbonate, which stabilizes mood in up to two-thirds of bipolar patients (Tondo et al. 1998). The antiepileptic drugs sodium valproate (valproic acid) (Freeman et al. 1992, Bowden et al. 1994), carbamazepine (Post 1990, Simhandl et al. 1993) and lamotrigine (Walden et al. 1996, Kusumakar and Yatham 1997, Ferreir 1998) are also effective, and have been increasingly used in bipolar patients as mood stabilizers (Figure 1). Adjunctive therapies include antidepressants (Yatham et al. 1998), antipsychotics (Ghaemi and Goodwin 1999), calcium channel blockers (Dubovsky 1993, Hollister and Trevino 1999) and other anti-convulsants (Marcotte 1998, Magnus 1999).

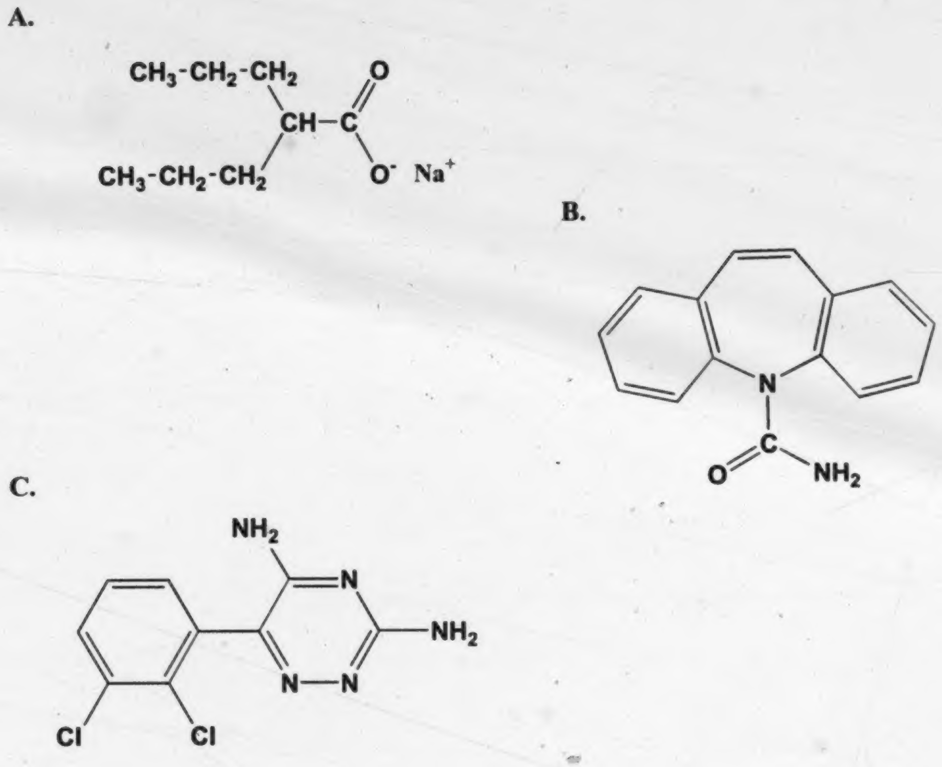

Figure 1 - Structures of Mood Stabilizers
Sodium valproate (A), carbamazepine (B) and lamotrigine (C).

Cellular signaling pathways have been proposed as candidates for the underlying neuronal pathophysiology of bipolar disorder, and as targets for mood stabilizers. These proposals began with the discovery that in the cerebral cortex of the rat, acute *in vivo* administration of lithium resulted in a 30% depletion of *myo*-inositol concentrations (Allison and Stewart 1976) and an accumulation of inositol-1-phosphates (I-1-P) (Allison et al. 1976). Moreover, incubation of rat cerebral cortical slices with lithium resulted in an accumulation of I-1-P, an effect that was markedly accentuated by agonists of receptors coupled to the phosphatidylinositol (PI-) cycle (Berridge et al. 1982). From these findings, the inositol-depletion hypothesis was proposed (Berridge et al. 1982, Berridge et al. 1989), which states that the therapeutic effectiveness of lithium is due to its ability to attenuate the responsiveness of the PI-second messenger pathway (Figure 2). Lithium acts on the PI-cycle by an uncompetitive inhibition of the enzymes inositol monophosphatase (IMPase) and inositol polyphosphate-1-phosphatase (IPPase), which are crucial within the PI-pathway for the regeneration of inositol phosphates (IP) (Hallcher and Sherman 1980, Nahorski et al. 1986, Majerus et al. 1988). It has been further hypothesized that the underlying abnormality in bipolar patients may be a hyper-responsive PI-cycle in neurons of one or more regions of the brain. Since the discovery that lithium acts on the PI-cycle, the study of the possible role of second messengers in psychiatric illness has broadened to include other components of the PI-cycle (Ishima et al. 1993, Gur et al. 1996, Manji et al. 1996), other second messenger pathways (Rahman et al. 1997, Fields et al. 1999, Perez et al. 1999) and other types of psychiatric disorders (Kaiya 1992, Strunecka and Ripova 1999).

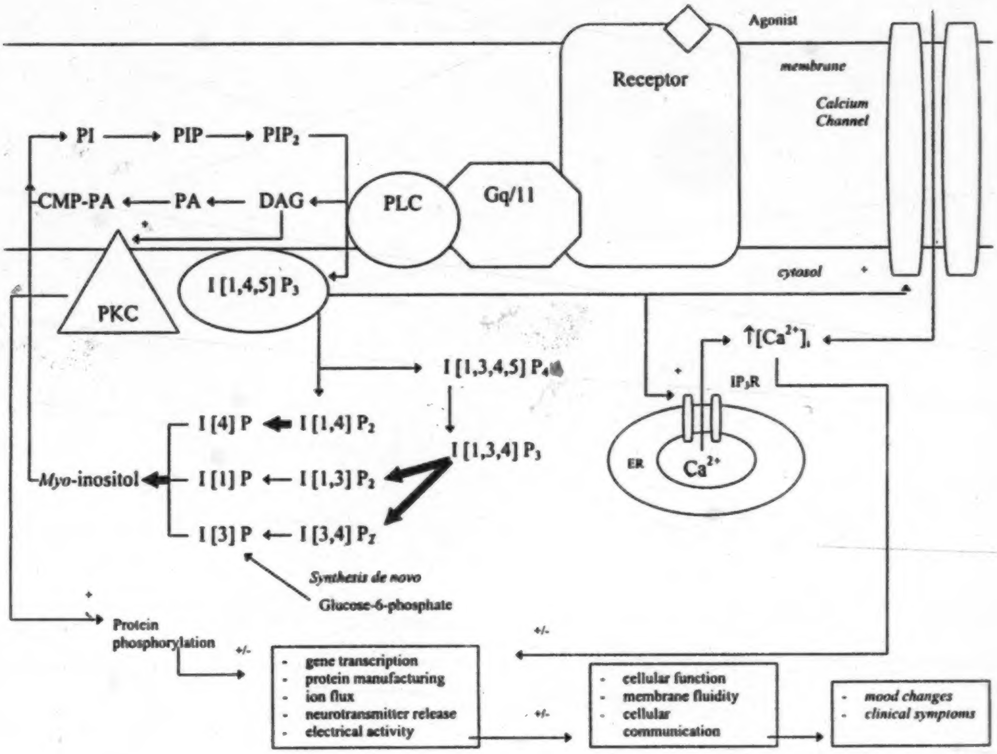
Agonist
membrane
Receptor
Calcium Channel
PI
PIP
PIP₂
CMP-PA
PA
DAG
PLC
Gq/11
PKC
I [1,4,5] P₃
cytosol
+
↑[Ca²⁺]ᵢ
I [1,3,4,5] P₄
IP₃R
ER
Ca²⁺
I [4] P
I [1,4] P₂
I [1,3,4] P₃
Myo-inositol
I [1] P
I [1,3] P₂
I [3] P
I [3,4] P₂
Synthesis de novo
Glucose-6-phosphate
Protein phosphorylation
+/-
- gene transcription
- protein manufacturing
- ion flux
- neurotransmitter release
- electrical activity
- cellular function
- membrane fluidity
- cellular communication
- mood changes
- clinical symptoms

Figure 2 - Phosphatidylinositol (PI-) Cycle

See text in sections 1.1 and 1.2 for a detailed description of illustration. Bold arrows indicate steps inhibited by lithium. PLC phospholipase C; PI phosphatidylinositol; PIP phosphatidylinositol phosphate; PIP_2 phosphatidylinositol bisphosphate; DAG sn-(1,2)-diacylglycerol; PA phosphatidic acid; CMP-PA cytidine monophosphate phosphatidate (also CDP-DG or cytidine diphosphate diacylglycerol); PKC protein kinase C; $I[x,x,x]P_3$ inositol trisphosphate; $I[x,x]P_2$ inositol bisphosphate; I[x]P inositol monophosphate; $I[x,x,x,x]P_4$ inositol tetrakisphosphate; $G_{q/11}$ G-protein subtype; ER endoplasmic reticulum; IP_3R inositol trisphosphate $I[1,4,5]P_3$ receptor; Ca^{2+} calcium ions; + activates; - inhibits.

1.2 The Phosphatidylinositol (PI-) Cycle

The PI-cycle is a second messenger pathway that is initiated after the binding of an agonist to a membrane receptor (Figure 2). Several receptors are linked to activation of the PI-cycle in neural tissues including receptors for serotonin (5-HT), epinephrine (E), acetylcholine (Ach) and dopamine (DA) (Nahorski et al. 1986, Fisher et al. 1992). Following the binding of an agonist to a receptor, the activated receptor undergoes a conformational change and binds to a G-protein. G-proteins are a family of heterotrimeric membrane proteins composed of α, β and γ subunits, which link receptors to enzymes responsible for the catalytic formation of cellular messengers (Lambert 1993). $G_{q/11}$ proteins are a sub-family of G-proteins that specifically couple receptors to the IP signaling cascade (Exton 1993). Upon receptor activation, a guanine triphosphate (GTP) molecule is exchanged for a guanine diphosphate (GDP) molecule on the $G\alpha$ sub-unit of the $G_{q/11}$-protein, causing the dissociation of $G\alpha$ from $G\beta\gamma$ sub-units (Exton 1993). The activated subunits further bind to and activate a specific phospholipase C (PLC) isozyme (Exton 1993). PLC then hydrolyzes phosphoinositol-(4,5)-bisphosphate (PIP_2) into the second messengers inositol-(1,4,5)-trisphosphate (IP_3) and *sn*-(1,2)-diacylglycerol (DAG) (Hughes and Putney 1988). IP_3 and DAG are metabolized and recycled back into the plasma membrane for the regeneration of membrane phosphoinositides (Irvine 1986). IP_3 is catabolised in the cytosol by IPPase and IMPase to form inositol bisphosphates (IP_2), I-1-P and *myo*-inositol, successively. DAG is broken down in the plasma membrane to phosphatidic acid (PA) which is then converted into cytidine monophosphate phosphatidate (CMP-PA) [also referred to as cytidine diphosphate diacylglycerol (CDP-DG)] (Berridge 1984). *Myo*-inositol and CMP-PA combine to reform PI in the plasma membrane (Berridge and Irvine 1989).

IP_3 and DAG further modulate cellular events by inducing an increase in cytosolic calcium ions (Ca^{2+}) from both intracellular and extracellular stores (Berridge 1993) and by activating protein kinase C (PKC) (Huang 1989), respectively (Berridge 1984). A rise in cytosolic Ca^{2+} triggers the release of neurotransmitters, and modulates gene transcription and protein synthesis (Berridge 1994). Intracellular Ca^{2+} levels are tightly controlled within the cell by a variety of Ca^{2+} channels, such as agonist-gated, voltage-gated and second messenger-gated channels, located in the plasma membrane or in the membranes of intracellular organelles (Kostuyuk and Verkhratsky 1994). The regulation of Ca^{2+} homeostasis is crucial due to a broad range of Ca^{2+}-dependent cellular processes, which can significantly influence cellular functioning. PKC phosphorylates proteins, such as enzymes and ion channels, to regulate their activity and modulate cellular ion flux (Buchner 1995). There are three main classes of PKC including: a conventional or Ca^{2+}-dependent (cPKC), a Ca^{2+}-independent (nPKC), and an atypical or Ca^{2+}-and phorbol ester-independent (Quest et al. 1996). Within each class, different isozymes of PKC are split between an "inactive pool" (cytosolic or soluble forms) and an "active pool" (membrane associated or particulate forms) (Casabona 1997).

Since changes in both intracellular Ca^{2+} and PKC can alter gene transcription, these second messengers can lead to long-term changes in cellular functioning. Thus, any abnormality in the PI-cycle signal has the potential to manifest itself in short-term and long-term cellular pathology. With regard to bipolar disorder, the presence of these abnormalities in specific brain regions may be ultimately manifest as psychological or behavioral disturbances.

1.3 Methodological Approaches

Approaches used to measure PI-cycle activity in mood disorders include: (i) the use of animals to investigate the effects of mood stabilizers on brain; (ii) *in vivo* nuclear magnetic resonance spectroscopy (MRS) of bipolar patients or healthy controls treated with mood stabilizers; (iii) post-mortem brain analysis of bipolar patients; and (iv) peripheral blood cell measures from bipolar patients or healthy controls treated with mood stabilizers. The elucidation of the effects of mood stabilizers on PI-cycle components and PI-cycle measurement in bipolar patients may indicate which biological abnormalities are present in bipolar disorder. The study of cellular messengers in the clinical setting is methodologically difficult, as the cells of interest in bipolar disorder are in the brain and not easily accessible. This difficulty has been approached by the implementation of biological and psychological models, two of which will be discussed here.

1.3.1 Peripheral Blood Cell Models

Peripheral blood cells, such as platelets and lymphocytes, are often used as neuronal models in psychiatric and brain research since they allow a non-invasive measurement of intracellular components in the clinical setting. These cells are often used in the study of bipolar disorder where there is such a focus on intracellular signaling pathways. A number of functional similarities exist between blood cells and neurons which validate, to some degree, their use as neuronal models (Sneddon 1973, Aronstam et al. 1977, Gershon et al. 1977, Omenn and Smith 1978, Weinshilboum 1978, Niggli et al. 1981, Rotman 1983, Stahl 1985, Brodde et al. 1986, Hashimoto et al. 1992, Authi

1993, Sage 1997). For example, platelets possess $5HT_2$ and α-adrenergic receptors (Sneddon 1973, Stahl 1985), 5-HT and DA re-uptake proteins (Omenn and Smith 1978), Ca^{2+} channels (Hashimoto et al. 1992, Authi 1993, Sage 1997), monoamine oxidase B (MAO_B) (Gershon et al. 1977) and binding sites for the antidepressant drug imipramine (Stahl 1985). Platelets store and release 5-HT in a Ca^{2+}-dependent manner (Sneddon 1973, Stahl 1985), they also express second messenger pathways, which lead to their aggregation for blood clotting and inflammation (Blockmans 1995). The activation of the PI-cycle in the platelet has many parallels to the activation of the PI-cycle in neurons (Siess 1989, Blockmans 1995, Guse 1998). Several endogenous agonists, such as E, NE, 5-HT, Ach, prostaglandins (PG) and cholecystekinin (CCK), are linked to the PI-cycle in both platelets (Siess 1989, Blockmans 1995, Mons and Cooper 1995), and neurons (Fisher et al. 1992), and are thus frequently used as research tools to stimulate second messenger pathways in blood cells. Blood-cell specific agonists also stimulate the PI-cycle, and include platelet activating factor (PAF), and thrombin in platelets, phytohemagluttinin (PHG) in lymphocytes, and formylmethionylleucylphenylalanine (fMLP) in neutrophils. Non-endogenous agonists can also activate pathways in both neurons and blood cells. Of these, isoproterenol, which binds to β-adrenergic receptors, is commonly used. Agonists which bind directly to $G\alpha$ sub-units, such as sodium fluoride (NaF), aluminum fluoride (AlF_4) and guananine triphosphate-γ-sulphate (GTPγS), are also used.

Due to the ubiquitous distribution of several cellular signaling components in cells throughout the body, and the functional similarities in cellular signaling machinery between neurons and blood cells, it is possible that changes in the PI-cycle in neurons linked to illness or induced by medication, may be affected similarly in peripheral blood cells. Thus peripheral blood cells provide a non-invasive measure which may give some indication of the central PI-cycle activity in bipolar disorder.

1.3.2 Dextroamphetamine as a Model of Mania

Pharmacological agents that induce psychological symptoms similar to those seen in mania (Peet and Peters 1995) or depression (Patten and Love 1997) may give insight into the underlying mechanisms of bipolar disorder and are thus used as research tools. Dextroamphetamine, a psychostimulant, (Figure 3) is often used as a model of mania in the study of bipolar disorder as it induces a manic-like syndrome which includes symptoms of euphoria, racing thoughts, increased talkativeness, goal-directedness, self-esteem, distractibility, and decreased need for sleep (Mamelak 1978, Jacobs and Silverstone 1986). Evidence that dextroamphetamine produces biological effects similar to those found in manic patients, e.g. increased heart rate, blood pressure (Jacobs and Silverstone 1986), catecholamine activity (Diehl and Gershon 1992) and cerebral metabolism (Vollenweider et al. 1998), suggests that it may also model some of the biological abnormalities of mania. Dextroamphetamine is thought to act mainly by inhibiting the reuptake of DA and NE in the brain. Other effects may include increasing the release of DA and NE and directly binding to post-synaptic receptors (Seiden et al. 1993, Kuczenski and Segal 1994). Since these effects lead to the activation of receptors linked to the PI-cycle (Fischer et al. 1992), dextroamphetamine may be useful in determining how the psychological symptoms of mania are linked to the PI-cycle.

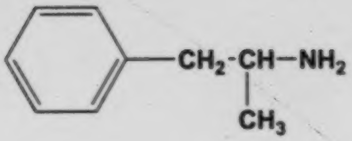

Figure 3 - Structure of Dextroamphetamine

1.4 Mood Stabilizers Affect the PI-Cycle – Animal Studies

1.4.1 Inositol-Depletion Hypothesis

The original depletion of inositol (Allison and Stewart 1971) and accumulation of I-1-P (Allison et al. 1976) reported from brains of lithium-treated rats has been replicated *in vivo* by a number of investigators (Table 1). The depletion of *myo*-inositol has been shown in lithium-treated rats under basal conditions (Sherman et al. 1981, Sherman et al. 1985, Hirvonen 1991, Hirvonen and Savolainen 1991) and after stimulation with centrally acting agonists (Sun et al. 1992). An increased formation of I-1-P was measured in lithium-treated rats under basal conditions (Sherman et al. 1981, Sherman et al. 1985, Savolainen et al. 1990, Hirvonen 1991, Preece et al. 1992, Lubrich et al. 1997) and after stimulation with centrally acting agonists (Whitworth et al. 1990, Atack 1992, Sun et al. 1992, Lin et al. 1993). However, not all investigators have reproduced these findings, since an increase in *myo*-inositol (Whitworth and Kendall 1989) and no change in *myo*-inositol formation (Lubrich et al. 1997) have also been reported (Table 1). A decreased formation of I-1- P under basal conditions (Li et al. 1993) and after agonist stimulation (Casebolt and Jope 1987, Elphick et al. 1988, Godfrey et al. 1989, Whitworth and Kendall 1989, Li et al. 1993), as well as no change in stimulated I-1-P formation (Honchar et al. 1990), have also been reported.

Several studies have confirmed and characterized the molecular and kinetic aspects of the uncompetitive inhibition of IMPase (Nahorski et al. 1991, Parthasarathy et al. 1994, Atack et al. 1995) and of IPPase by lithium (Hallcher and Sherman 1980, Majerus et al. 1988). In contrast to lithium, carbamazepine stimulated IMPase activity isolated from bovine brain, while valproate had no effect (Vadnal and Parthasarathy 1995). In view of these findings, and the knowledge of the diverse chemical properties of these drugs (Gagneux 1976, Arroyo and Sander 1999, Loscher 1999), it is likely that the therapeutic mechanisms of these anticonvulsants involve different steps of the PI-cycle than those affected by lithium. It has recently been shown that lithium, valproate and carbamazepine all inhibit the high affinity re-uptake of inositol into astrocytes (Lubrich and van Calker 1999). Although the penetration of inositol into the brain is thought to be low and dependent on slow inositol transporters (Spector 1988), the inhibition of inositol re-uptake into cells could result in a decreased availability of inositol over long periods of time, and thus be a potential mechanism by which these drugs decrease the responsiveness of the PI-cycle (Lubrich and van Calker 1999).

Brain region	Lithium		Agonist	*Myo*-inositol	I-1-P	Reference
	Treatment	Dose				
Cerebral cortex	Acute sc 5 hours	5 meq/kg	no	↓	↑	Allison et al. 1976
Cerebral cortex	Acute, ip 4-24 hours	6 meq/kg	basal mecamylamine pilocarpine	↓ ↓ ↓	↑ ↑ ↑	Sun et al. 1992
Cerebral cortex	Acute, ip 6-24 hours	10 meq/kg	no	↓	-	Allison and Stewart 1971
Frontal cortex, piriform cortex, caudate, thalamus, hippocampus, cerebellum	Acute, ip 24 hrs	5-18 meq/kg	no	↓ (not piriform cortex)	↑	Hirvonen 1991
Various regions	Acute, ip 0.5-24 hours	2.5 meq/kg	no	N	↑	Hirvonen and Savolainen 1991
Various regions	Acute, ip 0.5-24 hours	10 meq/kg	no	↓	↑	Hirvonen and Savolainen 1991
Frontal cortex, piriform cortex, caudate, thalamus, hippocampus, cerebellum	Acute, ip or sc 24 hours	5 meq/kg	no	N	↑	Savolainen et al. 1990
Cerebral cortex	Acute, sc 24 hours	10 meq/kg	no	↓	↑	Sherman et al. 1985
Whole brain	Acute, sc 24 hours	3.6, 7.2, 10.8 meq/kg	no	↓ (10.8 meq/kg)	↑ (10.8 meq/kg)	Preece et al. 1992

Cerebral cortex	Acute, ip 24 hours	10 meq/kg	5-HT carbachol norepinephrine	- - -	↓ N N	Godfrey et al. 1989
Frontal cortex, striatum	Acute, ip 18 hours	6.75 meq/kg	basal carbachol norepinephrine	- - -	N N N	Li et al. 1993
Cerebrum (mouse)	Acute, ip 4 hours	8 meq/kg	decapitation-ischemic-insult	-	↑	Lin et al. 1993
Caudate, cerebellum, cortex, hippocampus, hypothalamus	Acute, ip 24 hours	3,6,9 meq/kg	no	N	-	Lubrich et al. 1997
Cerebral cortex, hippocampus (mouse)	Acute, sc 18 hours	10 meq/kg	basal pilocarpine	- -	↑ ↑	Whitworth et al. 1990
Whole brain (mouse)	Acute, sc 6 hours	10 mmol/kg	basal pilocarpine	- -	↑ ↑	Atack et al. 1992
Cerebral cortex	Acute, sc 24 hrs	3 meq/kg	pargyline physostigmine pilocarpine	- - -	N N N	Honchar et al. 1990
Frontal cortex, striatum	Sub-acute, ip 3 days	2.5 meq/kg/day	basal carbachol norepinephrine	- - -	↓ (striatum) N N	Li et al. 1993
Various regions	Chronic, ip 1, 7 or 14 days	2.5 meq/kg/day	no	↓ (7 days, frontal cortex)	↑ (1, 7, days)	Hirvonen and Savolainen 1991
Cerebral cortex	Chronic, sc 9 days	3.6 meq/kg/day	no	N	↑	Sherman et al. 1981

Cerebral cortex	Chronic, sc 9 days	5.4, 7.2 meq/kg/day	no	↓	↑	Sherman et al. 1981
Cerebral cortex	Chronic, in food 14 days	3 meq/kg bid	5-HT carbachol norepinephrine	- - -	↓ ↓ ↓	Elphick et al. 1988
Cerebral cortex	Chronic, in food 30 days	1.7 g/kg of food	basal norepinephrine	- -	↓ ↓	Casebolt and Jope 1987
Cerebral cortex, hippocampus (mouse)	Chronic, in food 14 days	0.4% w/w of food	basal pilocarpine	- -	N ↑	Whitworth et al. 1990
Cerebral cortex, striatum	Chronic, in food 13 days	0.4 % w/w of food	carbachol	↑ (cortex)	↓ (cortex)	Whitworth and Kendall 1989
Caudate, cerebellum, cortex, hippocampus, hypothalamus	Chronic, in food 21 days	0.2% w/w of food	no	↓ (hypothalamus)	-	Lubrich et al. 1997
Cerebral cortex	Chronic, in food 28-52 days	40 meq/kg of food	pilocarpine pargyline physostigmine	- - -	N N N	Honchar et al. 1990
Cerebral cortex	Chronic, in food 22 days	40 mM/kg of food	no	↓	↑	Sherman et al. 1985
Frontal cortex, striatum	Chronic, ip 14 days	2.5 meq/kg/day	basal carbachol norepinephrine	- - -	↓ (frontal cortex) N N	Li et al. 1993
Frontal cortex, striatum	Chronic, ip 28 days	2.5 meq/kg/day	basal carbachol norepinephrine	- - -	↓(striatum) ↓ ↓ (frontal cortex)	Li et al. 1993

Cerebral cortex	Chronic, ip 28-52 days	2.5 meq/kg/day	pargyline	-	N	Honchar et al. 1990
			physostigmine	-	N	
			pilocarpine	-	N	
Cerebral cortex	Chronic, sc 3 or 16 day	3 meq/kg bid	5-HT	-	↓	Godfrey et al. 1989
			carbachol	-	↓	
			norepinephrine	-	↓	

Table 1 - Effects of *in vivo* Lithium Administration on *myo*-Inositol and I-1-P in Rat Brain

ip intraperitoneal injection; sc subcutaneous injection; meq/kg milliequivalents per kilogram of animal weight; mmol/kg millimoles per kilogram of animal weight; bid twice daily; 5-HT serotonin; ↑/↓/N increased, decreased or no change compared to control groups; - measure not reported.

1.4.2 Problems with Animal Studies

The inconsistencies in the animal findings are likely due to a number of variations in experimental conditions, which make generalizations about the effect of lithium on these PI-cycle components difficult. The studies summarized in Table 1 used various treatment doses, duration, routes and frequency of administration of lithium as well as different techniques to measure *myo*-inositol and I-1-P, all of which introduce potential sources of variation into the data. Differences in the type of agonist used, brain region studied, cell types and even rat strains (Savolainen et al. 1990), make it difficult to draw valid conclusions from between study comparisons, since variations in receptor population and regional sensitivity to the effects of lithium are expected. *Myo*-inositol depletion is usually found following high doses of lithium (> 5 meq/kg) (Allison and Stewart 1971, Sherman et al. 1981, Sherman et al. 1985, Hirvonen 1991, Hirvonen and Savolainen 1991, Sun et al. 1992). However, due to its toxic effects, these high doses of lithium cannot be given chronically to animals. Thus it is still unclear whether these acute affects of lithium are related to its long-term mood stabilizing properties. Chronic treatment with lower doses of lithium would be a better representation of the clinical situation, although past studies using this treatment regime have not reported any *myo*-inositol depletion (Hirvonen and Savolainen 1991, Sherman et al. 1991, Lubrich et al. 1997). However, these studies have not used receptor agonists, thus the inhibition of the PI-cycle produced by lithium may not be of great enough magnitude to be measurable. Indeed, studies using agonist stimulation have reported significant effects of lithium treatment on PI-cycle function (Elphick et al. 1988, Godfrey 1989, Whitworth and Kendall 1989, 1990).

While it is clear from these studies that lithium affects the PI-cycle, the animal experiments have not yielded results that are entirely consistent with the inositol-depletion hypothesis. Instead, they suggest that: (i) the effect of lithium on *myo*-inositol and I-1-P may be highly sensitive to the length and dose of drug treatment (specifically noted are differences between acute and chronic effects); (ii) different regions of the brain vary in their sensitivity and responses to lithium; and (iii) other mechanisms downstream of enzyme inhibition may be involved in lithium's therapeutic effect. Thus, while it is clear that lithium affects the PI-cycle, further investigation into these specific mechanisms and their relation to mood stabilizers is needed.

1.4.3 Does the Inositol-Depletion Hypothesis Apply to Other Mood Stabilizers?

Other mood stabilizers have also been reported to affect *myo*-inositol and IP in animal brain, suggesting that the PI-cycle may be a common target of mood stabilizers. Chronic valproate treatment increased stimulated I-1-P formation in the rat frontal cortex and significantly reduced stimulated I-1-P formation in the striatum (Li et al. 1993), while the incubation of mouse cerebral cortex slices with valproate had no effect on I-1-P (Dixon and Hokin 1997). Recently, we have found that chronic treatment of rats with valproate resulted in both a depletion of *myo*-inositol and an accumulation of inositol monophosphates in whole brain (O'Donnell et al. 2000). Acute carbamazepine treatment reduced electroconvulsive shock (ECS)-induced formation of I-1-P in the rat cortex and hippocampus (Vadnal and Bazan 1988). Incubation of rat hippocampal slices with carbamazepine resulted in a decreased formation of IP induced by carbachol, histamine and veratrin (a sodium channel agonist), but not by 5-HT or NE (McDermott and Logan

1989). Incubation of rat hippocampal astrocytes with carbamazepine resulted in an attenuation of phenylephrine-induced IP accumulation, which is proposed to be due to antagonism at the adenosine A1 receptor (Biber et al. 1996). However, chronic treatment with carbamazepine may result in an upregulation of these receptors (Biber et al. 1999). Formation of IP in rat cortex was not altered by *in vitro* incubation or by chronic *in vivo* treatment with carbamazepine with or without agonist stimulation (Manji et al. 1996). In summary, both valproate and carbamazepine affect the metabolism of IP, but the mechanisms by which this occurs are uncertain and may differ from those affected by lithium. While the inositol-depletion hypothesis may be specific for lithium, this hypothesis has led to studies which show that other mood stabilizers may have similar inhibitory effects to lithium on PI-cycle responsiveness. These findings indicate that the development of drugs that target the PI-cycle may be useful in treating bipolar disorder, and the use of PI-cycle inhibitors in bipolar patients is an exciting area for further study (Atack 1995, Levine et al. 1995, Kofman and Patishi 1999).

1.4.4 $I[1,4,5]P_3$, IP_2 and $I[1,3,4,5]P_4$

Lithium and the anticonvulsant mood stabilizers alter the concentrations of the inositol monophosphate precursor molecules, such as the inositol polyphosphates IP_3, IP_2 and IP_4. $I[1,4,5]P_3$, a second messenger largely responsible for Ca^{2+} mobilization in cells, is broken down to IP via IP_2 or $I[1,3,4,5]P_4$ and $I[1,3,4]P_3$ (Figure 2). In several regions of the rat brain, lithium decreased (Kennedy et al. 1989, Kennedy et al. 1990, Jope et al. 1992, Varney et al. 1992, Jenkinson et al. 1993, del Rio et al. 1998), increased (Whitworth et al. 1990, Dixon et al. 1992, Lee et al. 1992, Sun et al. 1992, Dixon et al.

1994) or had no effect (Sun et al. 1992, Ishima et al. 1993, Lin et al. 1993, Gur et al. 1996) on IP_3 concentrations. Three-week lithium treatment resulted in a greater concentration of IP_3 compared to one week of treatment (Ishima et al. 1993), suggesting that chronic lithium may have different effects than subacute lithium administration. Lithium may also increase (Kennedy et al. 1990, Lee et al. 1993), decrease (Kennedy et al. 1989, Kennedy et al. 1990) or have no effect (Dixon et al. 1992) on IP_4 concentrations, and increase the formation of IP_2 in rat brain (Sun et al. 1992, Lin et al. 1993, Dixon and Hokin 1997). Valproate increased the production of the Ca^{2+}-mobilizer $I(1,4,5)P_3$ but not that of $I(1,3,5)P_3$ or IP_2 in mouse cortical slices (Dixon and Hokin 1997). Acute carbamazepine injections attenuated the electroconvulsive shock (ECS)-induced formation of $[^3H]$-IP_3 in rat cortex and hippocampus (Vadnal and Bazan 1988). Although together these studies do not show consistent effects of any of the mood stabilizers on inositol polyphosphates, the important role of inositol polyphosphates in generating cellular effects by activation of the PI-cycle, and in the reformation of membrane phosphoinositides, warrants further study in this area. Additional investigations will help to elucidate the reasons for the observed variations between these studies and the long-term consequences of these effects at the cellular level.

1.4.5 Lithium Increases DAG, PA and CMP-PA and Decreases PI, PIP, PIP_2

Since mood stabilizer treatments result in changes in IP, it is likely that they will also change, whether directly or indirectly, the levels or activity of membrane phosphoinositides and membrane phospholipids, due to the interaction of these components with IP in the PI-cycle. Changes in the formation of PIP_2 ultimately lead to changes in the production of cytosolic second messengers, since PIP_2 is a substrate for PLC in the production of IP_3 and DAG. DAG is recycled back to PIP_2 via the formation of PA, CMP-PA, PI and PIP (Figure 2): indeed, mood stabilizers alter these components in animal brain. In cerebellar granule cultures, lithium initially potentiated potassium chloride depolarization-induced increases in DAG, then attenuated this response within 15 minutes (del Rio et al. 1998). Acute lithium also increased the formation of DAG in rat brain induced by NE, while carbamazepine had no effect (Kodama 1994). Lithium potentiated the accumulation of CMP-PA induced by carbachol in rat hippocampal, striatal and cortical slices (Godfrey et al. 1989, Jenkinson et al. 1993), and the accumulation of CDP-DG (CMP-PA) in neuroblastoma cells (Stubbs and Agranoff 1993). Lithium and carbachol also caused a 17-fold increase in CMP-PA in rat cerebellar granule cells when administered together, but neither had an effect alone (Gray et al. 1994). In the case of lithium administration, the accumulation of DAG and its metabolites may be explained by a depletion of *myo*-inositol, the substrate which combines with CMP-PA (CDP-DG) to form PI. Thus, a depletion of *myo*-inositol may lead to an accumulation of DAG, PA and CMP-PA in the cell membrane.

It is interesting to examine the effects of other mood stabilizers on the above components. Incubation of cortical synaptosomes with carbamazepine resulted in a decreased incorporation of ^{32}Pi into phospholipids, while valproate had no effect (Wei and Wang 1987). However, in rat brain, neither carbamazepine nor valproate had any effect on Ach-induced ^{32}Pi incorporation into PA (Aly and Abdel-Latif 1982), and acute carbamazepine had no effect on the formation of DAG induced by NE (Kodama 1994). These results suggest that valproate and carbamazepine do not share the same effect as lithium on the PI-cycle.

A decreased formation of PI, PIP, and PIP_2 is expected following a depletion of their common substrate (*myo*-inositol). This could result in an attenuation of the PI-cycle signal by leading to decreases in the production of DAG and IP_3. In fact, chronic lithium treatment resulted in decreased levels of PI in rat cortex (Joseph et al. 1987, Navidi et al. 1991) and hippocampus (Navidi et al. 1991). PI, PIP, and PIP_2 were all decreased in cultured cerebellar granule cells treated with lithium for two days, and stimulated with a receptor agonist (del Rio et al. 1996). In contrast, lithium treatment resulted in an increase of up to 60% in PIP_2 levels in rat hippocampal slices (Marinho et al. 1998). Acute carbamazepine accentuated ECS-induced PIP_2 formation in the rat cortex and hippocampus (Vadnal and Bazan 1988), although the mechanism by which this occurred is still unclear. The idea that the effect of lithium on these phosphoinositides may be specific to chronic exposure is supported by the findings that agonist-stimulated increases in PI, PIP or PIP_2 are *not* attenuated in whole rat brain after an acute intraperitoneal injection with lithium (Sun et al. 1992). However, increased (Godfrey et al. 1989, Navidi et al. 1991), decreased (Sun et al. 1992) and normal concentrations (Ishima et al. 1993) of basal PIP_2 have been reported in rats treated with lithium, suggesting that the magnitude and direction of this effect may also depend on the activity of the PI-cycle.

These studies provide further evidence that not only *myo*-inositol and I-1-P are affected by lithium and other mood stabilizers, but that the subsequent cascade of events in the PI-cycle is also affected. The findings suggest that lithium may indirectly change the membrane composition of cells through an inhibition of the PI-cycle. This phenomenon could in turn result in alterations in the transduction of agonist-induced signals via cellular membranes, which could result in abnormal cellular communication and homeostasis and eventually clinically detectable manifestations.

1.4.6 Chronic Lithium Treatment Attenuates $G_{q/11}$ *Protein Activity*

Although a number of studies have examined the function or activity of the cyclic-AMP pathway-coupled G-proteins (G_s and G_i) after treatment with mood stabilizers in rat brain (Colin et al. 1991, Lesch et al. 1991, Li et al. 1993c, Jakobsen and Wiborg et al. 1998), few studies have examined the effects of mood stabilizers on the function/activity of $G_{q/11}$ (PI-cycle specific) proteins. Variation in the number or activity of $G_{q/11}$ proteins could change the efficiency of the receptor coupling to the PI-cycle, and thus change the sensitivity of the PI-cycle to stimuli. A recent study reported that 5-HT-induced binding of [^{35}S]GTPγS to Gα_q was reduced by 38% in rat cortex after six weeks, but not after one week, of treatment with lithium (Wang and Friedman 1999). No differences were found in cortical G-protein levels, activity or coupling between rats untreated or treated with lithium for three weeks (Li et al. 1993c). However, NaF or GTPγS phosphoinositide hydrolysis and carbachol-induced potentiation of GTPγS were both significantly attenuated in the rat hippocampus, striatum and cortex after four weeks of lithium treatment, but not after *in vitro* incubation with lithium (Song and Jope 1992). These studies provide some evidence that lithium may affect $G_{q/11}$ protein activity more so after chronic use than with acute administration, which may relate to its long-term mood stabilizing properties.

1.4.7 Mood Stabilizers Attenuate PKC Activity

Since DAG and intracellular Ca^{2+} regulate PKC, changes in the PI-cycle by illness or medication could result in changes in PKC activity. To assess changes in PKC induced by mood stabilizers in rat brain, the following techniques have been used: radiolabeled-antibody electrophoresis, [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding and isozyme-specific immunolabeling (Table 2). Chronic lithium treatment reduced the activity of PKC in animal brain, as denoted by the reductions in particulate (or membrane associated) "active" PKC (Li et al. 1993a, Manji et al. 1993, 1996a, Bebcuck et al. 1998). Chronic valproate treatment produced a similar effect (Chen et al. 1994, Bebchuck et al. 1998). A decrease in the levels of both soluble and particulate myristolated alanine rich C kinase substrate (MARCKS), a PKC substrate, were also found with chronic lithium (Lenox et al. 1992, 1996) and chronic valproate treatment (Lenox et al. 1996, Watson et al. 1998). Similarly, a decrease in membrane-associated PKC substrates were found after chronic lithium (Casebolt and Jope 1991), chronic carbamazepine treatments (Jensen and Mork 1997) and in cerebral cortex slices after incubation with carbamazepine (Morishita and Watanabe 1994). Concurrent treatment with *myo*-inositol appears to often attenuate these effects of lithium on PKC activity (Lenox et al. 1996, Manji et al. 1996a), supporting the concept that the effect of lithium on PKC activity is brought about by an inhibition of the PI-cycle. Although still used as an experimental treatment for bipolar disorder, omega-3 fatty acids also inhibited PKC activity when incubated with PKC isolated from the rat colon (Holian and Nelson 1992). Together these results suggest that a common effect of chronic mood stabilizers may be an attenuation of PKC activity.

Studies which report no change in rat brain PKC levels or activity upon mood stabilizer treatment may be explained by the differences in brain regions studied, PKC isozyme specificity and duration of treatment (Table 2). Several studies found differences in PKC or related measures in rat brain after *chronic* treatment, without changes after *acute* treatment with mood stabilizers (Bebchuk et al. 1998, Lenox et al. 1992, 1996, Manji et al. 1993, Watson et al. 1998). Since the modulation of PKC activity appears to be slow, it is conceivable that this effect may contribute to the delayed therapeutic efficacy of mood stabilizers found in bipolar patients. In fact, the effect of mood stabilizers on PKC may be related to the mechanism of long-term onset of their mood stabilization effect in patients (Manji et al. 1996b), a concept which will be further discussed with the patient studies.

Region	Drug Treatment	Measure	Reference
Cortex	chronic lithium	↑ PKC (S)	Lin et al. 1993
		↓ PKC (P)	
Frontal Cortex	chronic lithium	N PKC α, β (I/II), γ, δ, ε, ζ (P)	Manji et al. 1996a
		N PKC α, β (I/II), γ, δ, ε, ζ (S)	
		N MARCKS (P)	
		N MARCKS, GAP-43 (S)	
		↑ ADPR (vs *myo*-inositol alone)	
	chronic lithium +	↑ PKC β (I/II) (P)	
	myo-inositol	↑ GAP-43 (P, vs *myo*-inositol alone)	
Hippocampus	acute lithium	N MARCKS (S)	Lenox et al. 1992
	chronic lithium	↓ MARCKS (S)	
Hippocampus	acute lithium	N PKC (S and P, S:P ratio, activity)	Lenox et al. 1992
	chronic lithium	N PKC (S and P, S:P ratio, activity)	
Hippocampus	acute lithium	N PKC α, β, γ (S and P)	Manji et al. 1993
	chronic lithium	↓ PKC α (P)	
		N PKC α (S)	
		N PKC β, γ (S and P)	
Hippocampus	chronic lithium	↓ PKC substrate phosphorylation (P)	Casebolt and Jope 1991
		↑ PKC substrate phosphorylation (S)	
		N PKC (S and P)	

Hippocampus	chronic lithium	↓ PKC ε (P) N PKC β (I/II), δ, ζ (P) N PKC α, β (I/II), γ, δ, ε, ζ (S) N MARCKS, GAP-43 (S and P) ↑ ADPR	Manji et al. 1996a
	chronic lithium + *myo*-inositol	N PKC α (P, attenuation) N PKC ε (P, attenuation) N ADPR (attenuation)	
HL60 cells	chronic lithium (*in vitro)*	↓ PKC α (S and P)	Bitran et al. 1995
Limbic	acute lithium	N PKC α and ε (P)	Bebchuk et al. 1998
	chronic lithium	↓ PKC α and ε (P)	
Cerebral cortex	lithium (*in vitro)*	N PKC activity	Morishita and Watanabe 1994
	carbamazepine (*in vitro*)	↓ PKC activity	
Cortex	chronic lithium	N PKC substrate phosphorylation (P)	Jensen and Mork 1997
	chronic carbamazepine	↓ PKC substrate phosphorylation (P)	
C6 glioma cultures	chronic valproate	↓ PKC α and ε; N PKC δ and ζ (S and P) ↑ PKC (S:P ratio)	Chen et al. 1994
Hippocampus	chronic lithium	↓ PKC substrate phosphorylation (P)	Jensen and Mork 1997
	chronic carbamazepine	N PKC substrate phosphorylation (P)	
Hippocampal cell cultures	acute lithium	N MARCKS (S and P)	Lenox et al. 1996
	chronic lithium	↓ MARCKS (S more than P)	
	chronic lithium + valproate	↓ MARCKS (S and P, additive)	
	chronic lithium + *myo*-inositol	N MARCKS (S and P, attenuation)	

Hippocampal cell cultures	acute valproate	N MARCKS (S and P)	Lenox et al. 1996
	chronic valproate	↓ MARCKS (S and P)	
	chronic valproate + *myo*-inositol	↓ MARCKS (S and P)	
Hippocampal cell cultures	acute valproate	N MARCKS (S and P)	Watson et al. 1998
	chronic valproate	↓ MARCKS (P more than S)	
	chronic valproate + lithium/carbachol	↓ MARCKS (S and P, additive)	
Hippocampal cell cultures	acute carbamazepine	N MARCKS (S and P)	Lenox et al. 1996, Watson et al. 1998
	chronic carbamazepine	N MARCKS (S and P)	
Limbic	acute valproate	N PKC α and ε (P)	Bebchuk et al. 1998
	chronic valproate	↓ PKC α and ε (P)	

Table 2 - Effects of Mood Stabilizers on Protein Kinase C (PKC) and Related Substrates in Rats

S soluble or cytosolic fraction; P particulate or membrane fraction, MARCKS myristolylated alanine-rich C kinase substrate; GAP-43 growth associated protein; ADPR [^{32}P]adenosine diphosphate-ribosylation; ↑/↓/N increased, decreased or unchanged values compared to control groups unless otherwise specified.

1.5 PI-Cycle Abnormalities in Bipolar Patients

1.5.1 In Vivo Magnetic Resonance Spectroscopy of myo-Inositol and IP

Myo-Inositol and IP can be measured in the brain *in vivo* using proton (^{1}H) and phosphorus (^{31}P) nuclear magnetic resonance spectroscopy, respectively (Maier 1995, Kato et al. 1998, Kegeles et al. 1998). When coupled to magnetic resonance imaging, MRS allows non-invasive quantitative measurements of endogenous chemical nuclei in functional brain (Allen 1990, Bachelard and Badar-Goffer 1993). MRS utilizes randomly spinning elemental nuclei containing odd numbers of electrons, such as ^{1}H and ^{31}P. After application of a radiofrequency pulse in a static magnetic field, each nucleus resonates back to its resting state at characteristic frequencies, which are then converted into spectral signals (Figure 4).

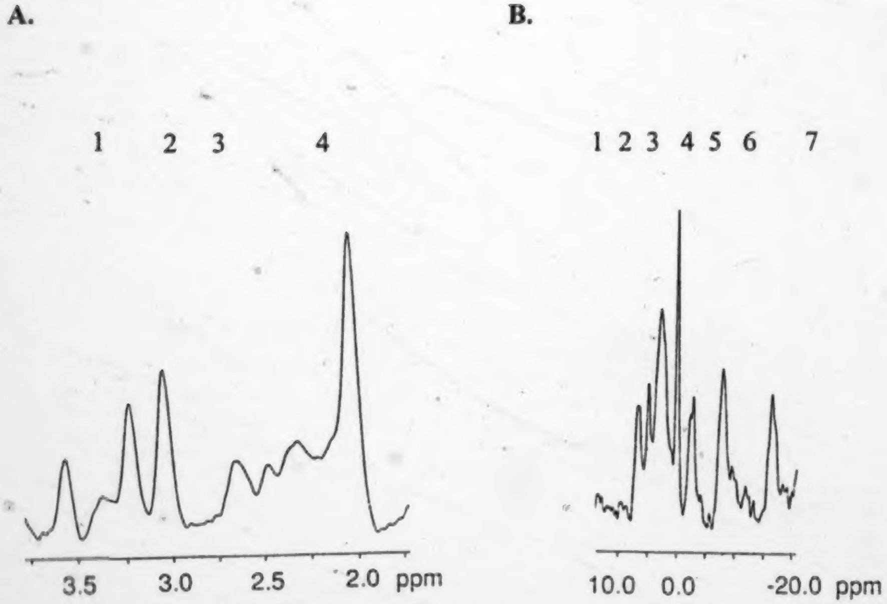

Figure 4 - *In Vivo* ^{1}H and ^{31}P Spectra

^{1}H (A) and ^{31}P (B) spectra *in vivo* MRS scanned from temporal lobe of healthy subjects. Peaks are (A): 1 – *myo*-inositol, 2 – choline, 3 – phosphocreatine/creatine, 4 – N-acetylaspartate; (B): 1 – phosphomonoesters (PME), 2 – inorganic phosphate, 3 – phosphodiesters, 4 – phosphocreatine, 5, 6, 7 – ATP (γ, α, β, respectively). Modified with permission from Silverstone et al. (1996).

The phosphomonoester (PME) peak contains I-1-P in the ^{31}P spectrum. The 3.65 and 3.54 peaks in the ^{1}H spectrum contain *myo*-inositol. However, due to the low-resolution strength of the *in vivo* magnet, these peaks are contaminated by co-resonating signals: the *myo*-inositol peaks contain the signals from glycine and I-1-P (Behar and Ogino 1991), while the PME peak also contains signals from phosphoethanolamine, phosphocholine and L-phosphoserine (Gyulai et al. 1984). In the ^{1}H, spectra N-acetylaspartic acid (NAA), choline and creatine are also measured and used as reference peaks. Other metabolites measured as reference peaks in the ^{31}P spectra include the phosphodiester (PDE) peak, which contains signals from glycerol-3-phosphocholine, glycerol-3-phosphoethanolamine and phosphoserine, and is generally considered as a measure of membrane phospholipid turnover (Murphy et al. 1989, Kilby et al. 1991); inorganic phosphate (Pi), phosphocreatine (PCr) and adenosine triphosphate (ATP). All of the above indicate that these measurements must be interpreted with caution since: (i) more than one compound may be represented by a single peak and (ii) drug or disease-induced changes in the reference peaks could lead to uncertainty in the reported measures of interest.

^{1}H MRS has been used to measure *myo*-inositol in both medicated and unmedicated bipolar patients in various mood states (Table 3). In concordance with the inositol-depletion hypothesis proposed from the animal experiments, a decrease of inositol was demonstrated *in vivo* in the frontal lobes of manic lithium-treated bipolar patients compared to their pre-treatment levels (Moore et al. 1999, Yue et al. 1999). A decrease in *myo*-inositol concentrations was also reported in the frontal lobe of a mixed group of unipolar and bipolar depressed patients taking various antidepressants compared with age-matched healthy controls (Frey et al. 1998). However, several studies have found no differences between lithium-treated euthymic patients and controls in the occipital cortex (Sharma et al. 1992), cerebral cortex (Bruhn et al. 1993) or temporal cortex (Silverstone et al. 2000, unpublished data). Furthermore, no differences in *myo*-inositol concentrations were found between healthy controls treated with placebo and those treated with lithium for one week (Silverstone et al. 1996), or those treated with lithium for one week followed by dextroamphetamine (Silverstone et al. 2000). Interestingly, an increase in *myo*-inositol has been observed in the basal ganglia of lithium-treated euthymic patients in one small study (Sharma et al. 1992). Thus, while the inositol-depletion hypothesis has yet to be consistently replicated *in vivo*, it is likely that, similar to the animal studies, this effect is sensitive to the dose and duration of lithium treatment and may be more detectable in some areas of the brain than others. It may also be that the effect of lithium is to normalize levels of *myo*-inositol, and thus no differences in *medicated* patients would be expected compared to healthy controls. Rather, differences might be expected when comparing levels of *myo*-inositol between unmedicated or acutely ill patients with medicated or euthymic patients, a study design which has been used recently by investigators reporting differences in *myo*-inositol between these groups (Moore et al. 1999, Yue et al. 1999).

The accumulation of I-1-P predicted from the inositol-depletion hypothesis has also been tested in lithium-treated patients by measuring the PME peak in ^{31}P spectra (Table 3). In medicated (mainly with lithium) euthymic patients, a *decrease* in PME concentrations has been reported in the frontal lobes compared to healthy controls (Kato et al. 1992, 1993, 1994). Correspondingly, the administration of dextroamphetamine as a model of mania (Jacobs and Silverstone 1986) increased PME in the temporal lobe of healthy volunteers (Silverstone et al. 2000, unpublished data). This effect was not seen in euthymic bipolar patients medicated with either lithium or valproate (Silverstone et al. 2000, unpublished data), or in healthy controls treated for one week with lithium alone (Silverstone et al. 1996, Silverstone et al. 2000). Additionally, PME concentrations in the frontal lobes in medicated euthymic bipolar patients were not different from those of healthy controls (Kato et al. 1992, 1994). Thus, in comparison with the *myo*-inositol data, a baseline abnormality in the PME peak may not be detected in euthymic or medicated patients, as this abnormality could be masked by an effect of clinical remission or drug treatment.

Studies in unmedicated and acutely ill bipolar patients lend support to the above claim that a trait abnormality of PME concentrations may exist in bipolar patients. One group has found a decrease in PME concentrations in the frontal lobes (Deicken et al. 1995a) and temporal lobes (Deicken et al. 1995b) of *unmedicated* euthymic bipolar patients. The concentration of PDE were increased in the frontal lobes of unmedicated euthymic patients (Deicken et al. 1995a, Kato et al. 1998b). PME concentrations may also vary with mood state since *increases* in PME concentrations have been found in both the frontal lobes of medicated manic patients (Kato et al. 1991, 1994b) and medicated depressed bipolar patients (Kato et al. 1992, 1994b, 1995), (in contrast to the decrease seen in the euthymic phase). An increase in PME was also found in medicated unipolar patients in the depressed state, suggesting that there may be some biological similarities between unipolar and bipolar depression (Volz et al. 1998), although normal levels have also been reported (Kato et al. 1992). A decrease in the PME peak and an increase in the

PDE peak has been reported in several brain regions of both medicated and unmedicated schizophrenic patients (Pettegrew et al. 1991, Stanley et al. 1995, Fukuzako et al. 1999, Volz et al. 1999), suggesting that a disruption in the PI-cycle may be present in other psychiatric disorders.

Taken together, these studies support the idea that the therapeutic mechanisms of mood stabilizers may be a result of their effects on the PI-cycle, but the patient findings somewhat contradict the animal findings. As in the initial animal studies, the effects of lithium may be more pronounced when the PI-cycle is stimulated with an agonist, such an effect produced by dextroamphetamine, and are likely regionally specific. With regard to the etiology of bipolar disorder, it is possible that *myo*-inositol and PME concentrations may be increased in acute episodes of bipolar disorder and normalized or even lowered below normal by lithium treatment. This would explain some of the contradictory findings, such as those observed in medicated euthymic patients, where *myo*-inositol levels were similar to and PME levels were lower than normal controls.

Subject	n	Region	Medication	*myo*-Inositol	PMEs and PDEs	Reference Peak	Reference
BP – manic	8	Frontal Lobe	lithium (1 week)	↓ (vs baseline)	-	/Cr absolute	Yue et al. 1999
BP - manic	12	Frontal Lobe, R & L	various	-	N PME	% TP	Kato et al. 1995
BP – manic	11	Frontal Lobe	lithium + various	-	↑ PME (vs HC and vs euthymics)	% TP	Kato et al. 1991
BP (II) – manic	15	Frontal Lobe	various	-	↑ PME	% TP	Kato et al. 1994
BP – euthymic	4	Basal Ganglia	lithium	↑	-	/PCr-Cr	Sharma et al. 1992
BP – euthymic	8	Cerebral Cortex	lithium	no change no change	-	/Cr absolute	Bruhn et al. 1993
BP – euthymic	17	Frontal Lobe	lithium	-	↓ PME	% TP	Kato et al. 1993
BP – euthymic	10	Frontal Lobe	lithium + various	-	↓ PME N PDE	% TP	Kato et al. 1992
BP – euthymic	31	Frontal Lobe	lithium + various	-	↓ PME	% TP	Kato et al. 1994
BP (II) – euthymic	15	Frontal Lobe	various	-	N PME	% TP	Kato et al. 1994
BP – euthymic	21	Frontal Lobe, L & R	various	-	N PME	% TP	Kato et al. 1995
BP – euthymic	12	Frontal Lobe, R & L	no (1 week)	-	↓PME ↑ PDE	% TP	Deicken et al. 1995
BP – euthymic	4	Occipital Lobe	lithium	no change	-	/PCr-Cr	Sharma et al. 1992

BP – euthymic	14	Temporal Lobe, L	lithium + acute amphetamine	no change	↓ PME	/β-ATP	Silverstone et al. 2000, unpublished data
BP – euthymic	19	Temporal Lobe, L	valproate + acute amphetamine	no change	↓ PME	/β-ATP	Silverstone et al. 2000, unpublished data
BP – euthymic	14	Frontal Lobe	no (10 days)	-	↑ PDE	% TP	Kato et al. 1998
BP – euthymic	12	Temporal Lobe	no (1 week)	-	↓ PME N PDE	% TP	Deicken et al. 1995
BP – depressed	10	Frontal Lobe, R	lithium (5-7 days or 3-4 weeks)	↓ (vs baseline)	-	absolute	Moore et al. 1999
BP – depressed	10	Temporal, L, Parietal Lobe, L, Occipital Lobe, C	lithium (5-7 days or 3-4 weeks)	no change (vs baseline)	-	absolute	Moore et al. 1999
BP – depressed	10	Frontal Lobe	lithium + various	-	↑ PME (vs euthymics) N PDE	% TP	Kato et al. 1992
BP (II) – depressed	15	Frontal Lobe	various	-	↑ PME	% TP	Kato et al. 1994
BP/UP – depressed	10	Frontal Lobe, R	antidepressants	↓	-	/Cr	Frey et al. 1998
BP – depressed	11	Frontal Lobe, L	various	-	↓ PME	% TP	Kato et al. 1995
UP – depressed	12	Frontal Lobe	antidepressants	-	N PME N PDE	% TP	Kato et al. 1992

UP – depressed	14	Frontal lobe	lithium + various	-	↑ PME N PDE	% TP	Volz et al. 1998
HC	7	Temporal Lobe	lithium (1 week)	no change	no change	/Cr /PCr	Silverstone et al. 1996
HC	10	Temporal Lobe	lithium (1 week) + acute amphetamine	no change	↑ PME ↑ PME	/Pi+PCr /β-ATP	Silverstone et al. 2000

Table 3 - *In vivo* ^{1}H and ^{31}P Nuclear Magnetic Resonance Spectroscopy (MRS) Measures of *myo*-Inositol and PMEs in Bipolar Disorder

BP bipolar disorder; HC healthy control; UP unipolar disorder (major depressive disorder); II bipolar II disorder; R right; L left; C central; various medications including mood stabilizers, antidepressants and antipsychotics; PME phosphomonoesters; PDE phosphodiesters; TP total phosphorus; PCr phosphocreatine; Cr creatine; Pi inorganic phosphorus; β-ATP beta-adenosine triphosphate; ↑/↓/N increased, decreased or normal compared to healthy controls unless otherwise stated; - measure not reported

1.5.2 Problems with In Vivo Human Studies

Caution needs to be applied when considering *in vivo* findings since, like the animal experiments, there are several discrepancies between studies. These differences can be attributed to a number of factors. First, the heterogeneity of the patient populations used in these studies raises question as to whether the effect reported is a trait of the disorder, associated with mood state, or an effect of medication. This also makes it difficult to make valid comparisons between studies. Second, inconsistencies may be due to differences in methods of data acquisition between studies, such as the type of pulse sequence used and magnet strength. The strength (1.5-3 Tesla) of the magnet used for *in vivo* NMR is much smaller than that used for *in vitro* NMR (12-14 Tesla). This lower strength results in lower resolution, signal overlap, lower signal-to-noise ratios and subsequently a higher standard deviation within study groups in comparison with *in vitro* data (Michaelis et al. 1991, Soares et al. 1996). Third, there may be regional differences in trait abnormalities or drug effects which make it difficult to compare results between studies that measure metabolites from different brain regions. Fourth, it is likely that the measurable effects of mood stabilizers on *myo*-inositol and PME change over time in human brain due to the adaptation of neurons to chronic drug administration and the additional interplay of homeostatic mechanisms. This suggestion is supported by *in vivo* measurements from the brains of cats treated with lithium for four weeks, which revealed that PME concentrations are initially increased, then decline to normal levels by three and four weeks (Renshaw et al. 1986).

Another problem with *in vivo* measurements is that, in the past, most of the data have been expressed in ratio format relative to other brain metabolites, rather than absolute quantities (as seen in Table 3). *Myo*-inositol peak areas are referenced to creatine or NAA while PME peak areas are referenced to total phosphorus signal, ATP or PCr. It has generally been assumed that these reference peaks are stable metabolites in the brain insensitive to disease or medication, but there is evidence that the levels of these metabolites are not stable and that these may not be valid reference peaks (Kato et al. 1994b, Winsberg et al. 1998, O'Donnell et al. 2000). Hence, the *in vivo* data referenced to these peaks may actually be confounded by changes in the reference peaks. Thus, while *in vivo* MRS measurements add valuable support to the involvement of the PI-cycle in bipolar disorder, the methodological limitations require that caution be taken in interpreting these findings, and that conclusive explanations cannot yet be generated from these studies. The further development of *in vivo* MRS methodology and more rigorous study designs will improve the quality of the research generated in this area.

1.5.3 Ex Vivo Measurements of myo-Inositol and IP

Analysis of post-mortem brain tissue from bipolar patients lends some support to the inositol-depletion hypothesis and to the suggestions of an abnormal PI-cycle function. While there is the possibility that post-mortem tissue changes and variable causes of death will confound this type of data, studies of the components of the PI-cycle studied to date in post-mortem bipolar patient brains have generated relatively stable results within a post-mortem time interval of at least five hours (Greenwood et al. 1995). Post-mortem bipolar patient brains displayed normal IMPase activity (Shimon et al. 1997), while in contrast, a decrease in IMPase levels was detected post-mortem in the frontal and occipital cortices and in the cerebellum of schizophrenic patients (Shimon et al. 1998). It is conceivable that the effect of the medication was to normalize IMPase activity in bipolar patients, which may have been increased above normal before the administration of the medication. The abnormally low level of IMPase activity in schizophrenia could be related to a reduced size, number or development of neurons associated with schizophrenia (Harrison 1999).

Reduced *myo*-inositol concentrations were found post-mortem in the frontal lobe and hypothalamus of lithium-treated euthymic bipolar patients (Belmaker et al. 1998) and in the frontal lobe of medicated depressed bipolar patients who committed suicide (Shimon et al. 1997), compared with healthy controls. This effect may be regionally sensitive, as normal *myo*-inositol concentrations were reported in the cerebellum and occipital lobes of the latter group. It should be noted, however, that these post-mortem changes may not be specific to bipolar disorder since a decrease in post-mortem *myo*-inositol concentrations below that of normal controls has also been found in the occipital and frontal cortices and the cerebellum of post-mortem schizophrenic patients (Shimon et al. 1998). The formation of phosphoinositides in the occipital cortex of medicated bipolar patients in response to either carbachol or 5-HT was decreased compared with healthy controls, giving further support to the suggestions that mood stabilizers attenuate PI-cycle sensitivity to stimuli (Jope et al. 1996). As with the *in vivo* MRS studies, it is difficult to draw conclusions because different brain regions, medications or patient groups have been examined in these studies. Thus, while these differences are interesting and suggest that an abnormal PI-cycle function is associated with bipolar disorder, the causes and implications of these changes are still unclear.

Data from peripheral blood cells generally support the inositol-depletion hypothesis of lithium's actions. Neutrophils from a mixed group of male bipolar, major depression or schizophrenic patients all on chronic lithium therapy exhibited a significantly attenuated fMLP-induced rise in cytosolic [^{3}H] *myo*-inositol and IP_3 compared with age- and sex-matched healthy controls and female patients (Greil et al. 1991). The activity of IMPase isolated from platelets of lithium-treated manic patients, as measured by IP production, was significantly lower than healthy controls (Moscovich et al. 1990). In another study, the IMPase activity from erythrocytes of healthy controls was inhibited by incubation with therapeutic lithium concentrations (Agam and Livne 1989). The data give additional support to the inositol-depletion hypothesis of lithium, and are in agreement with the findings in animals that lithium inhibits IMPase activity. Interestingly, the efficacy of omega-3 fatty acids in the adjunctive treatment of bipolar disorder (Shimon et al. 1997, Stoll et al. 1999) may be related to their ability to attenuate the PI-cycle. The formation of IP was attenuated in stimulated peripheral blood cells taken from healthy volunteers (Sperling et al. 1993) and rabbits (Medini et al. 1990) after chronic treatment with omega-3 fatty acids.

1.5.4 Post-Mortem Brain PLC is Not Changed

An abnormal activity of PLC could result in abnormal PI-signaling, and has thus been studied in post-mortem brain tissue taken from bipolar patients. However, normal PLC activity has been reported in the frontal, temporal and occipital cortices of post-mortem brain from medicated bipolar patients (Jope et al. 1996, Mathews et al. 1997), in the prefrontal cortex of both medicated and unmedicated patients with major depressive disorder, (Pacheco et al. 1996), as well as in the frontal cortex of medicated schizophrenic patients (Jope et al. 1998). There was a non-significant trend to increased levels of PLC-β, the isoform linked to the PI-cycle, in the occipital cortex of medicated bipolar patients (Mathews et al. 1997). Thus, while the evidence from post-mortem studies has not generated data to suggest the direct involvement of PLC in bipolar disorder, it is possible that PLC abnormalities may be masked by chronic medication use.

1.5.5 Increased Levels of Peripheral Cellular PIP$_2$ in Mania are Normalized with Lithium Treatment

Measures of phosphoinositide activity in blood cells from bipolar patients suggest that a hypersensitive or overactive PI-pathway may be a characteristic of these patients. Levels of membrane phospholipids have been measured in platelets using two-dimensional thin layer chromatography coupled with two-dimensional scanning laser densitometry (Mallinger et al. 1993). With this method, platelet membrane PIP$_2$ has been reported significantly higher in unmedicated bipolar patients in the manic state than in healthy controls or euthymic bipolar patients (Brown et al. 1993, Soares et al. 1997), and significantly lower in lithium-treated euthymic patients (Soares et al. 1999a). Moreover, membrane PIP$_2$ levels in platelets of a single bipolar patient in whom PIP$_2$ levels increased in the manic phase normalized with return of the patient to the euthymic state following lithium treatment (Soares et al. 1997). PIP$_2$ levels were also decreased after three weeks of lithium treatment in a group of initially unmedicated, acutely ill, bipolar patients (Soares et al. 1999b). These findings suggest that PIP$_2$ may be abnormally elevated in mania and normalized by lithium treatment. A decreased activity of PLC has also been found in platelets from lithium-treated euthymic bipolar patients (Ebstein et al.

1988). Thrombin-induced formation of CMP-PA was enhanced in platelets of healthy volunteers after incubation with lithium (Watson et al. 1990), a finding consistent with the effects of lithium in animal brain. However, the specificity of these changes to bipolar patients is uncertain since an increase in PIP_2 levels has also been found in both stimulated and unstimulated platelets of schizophrenic patients taking neuroleptics compared to healthy controls (Das et al. 1994), suggesting that a similar abnormality in phosphoinositides may also be present in schizophrenia. Changes in membrane phospholipids and phosphoinositides have also been implicated in the pathophysiology of schizophrenia (Horrobin et al. 1994), and thus these membrane components may be a therapeutic target of nutritional therapies, such as omega-3 fatty acids, in both schizophrenia and bipolar disorder (Peet et al. 1996).

1.5.6 Ex Vivo Measurements of $G_{q/11}$ Proteins are Abnormal

Although one study has reported an increase in the $G\alpha_{q/11}$ subunit in the occipital cortex of bipolar patients taking various medications (Mathews et al. 1997), immunolabeling techniques revealed normal concentrations of $G_{q/11}$ proteins in post-mortem brain tissue from bipolar patients in both the frontal cortex (Friedman and Wang 1996, Mathews et al. 1997) and temporal cortex (Mathews et al. 1997). Similarly, normal levels of $G_{q/11}$ have also been found in post-mortem frontal cortex in schizophrenia (Jope et al. 1998) and in prefrontal cortex in major depressive disorder (Pacheco et al. 1996).

In comparison, the *activity* of $G_{q/11}$-proteins measured by the extent of phosphoinositide hydrolysis following direct stimulation of cells with GTP-γ, a directly acting G-protein agonist, was decreased in two studies. Post-mortem $G_{q/11}$-protein activity was decreased below normal levels in the occipital cortex of bipolar patients medicated with lithium and adjunctive mood stabilizers (Jope et al. 1996), and in the prefrontal cortex of both medicated and unmedicated patients with major depressive disorder, who had committed suicide (Jope et al. 1996). In contrast, the activity of the $G\alpha_q$ subunit was increased in the frontal cortex of bipolar patients (Friedman and Wang 1996). Interestingly, $G_{q/11}$ protein activity was increased in the frontal cortex of post-mortem schizophrenic brains (Jope et al. 1998). Taken together, these results suggest that a decrease in the coupling efficiency of $G_{q/11}$-proteins to phosphoinositide hydrolysis could be a common trait of affective disorders or an effect of mood stabilizers, while an increase in the activity of PI-cycle specific $G_{q/11}$-proteins could be associated with schizophrenia.

In bipolar disorder, platelets and lymphocytes of lithium-treated euthymic patients exhibited significantly lower levels of $G_{q/11}$ proteins, but these were normal in unmedicated manic patients (Manji et al. 1995). In contrast, the levels of $G\alpha_q$ were similar in both platelets and lymphocytes from medicated bipolar patients compared to healthy controls (Mitchell et al. 1997). $G_{q/11}$ proteins have also been examined in unipolar depression, studies that are important to consider due to the symptom overlap with bipolar depression. The increase in $G\alpha_q$ found in platelets of unmedicated patients with unipolar depression was significantly decreased following treatment with antidepressants and improvement of clinical symptoms (Karege et al. 1998). The receptor coupling efficiency of G-proteins, assessed by an enhanced receptor-stimulated rise in platelet IP using thrombin or E, increased in unipolar depressed patients compared to healthy controls, but there were no differences after direct G-protein activation with NaF (Karege et al. 1996). In contrast, normal $G_{q/11}$ levels were present in platelets and leukocytes from unmedicated patients with panic disorder (Stein et al. 1996). These studies do not suggest that the primary cause of bipolar disorder is related to levels of $G_{q/11}$-protein dysfunction; instead mood stabilizers may down-regulate $G_{q/11}$ protein *activity* as a result of their effects on the PI-cycle. This phenomenon is supported by the animal studies, which show that mood stabilizers down-regulate $G_{q/11}$ activity and warrants further studies of $G_{q/11}$ protein activity in patients.

1.5.7 Ex Vivo Measurements of PKC Activity Indicate Variations with Mood in Affective Disorders and Sensitivity to Mood Stabilizers: Increased PKC Activity in Mania is Attenuated by Mood Stabilizers

Post-mortem studies of PKC in bipolar patient brain tissue have also revealed changes in PKC, although the cause of these changes are uncertain due to the mixed use of patient medications (Table 4). The levels of the cytosolic α isozyme and the membrane associated γ and ζ isozymes of PKC were increased in the cortex of brains obtained post-mortem from medicated bipolar patients compared to healthy controls (Wang and Friedman 1996). These bipolar patients also exhibited a significantly potentiated redistribution of the cytosolic (inactive) form of PKC to the membrane-associated (active) form of PKC upon activation with a PKC agonist, phorbol 12-myristate 13-acetate (PMA), compared to healthy controls (Wang and Friedman 1996). In contrast, the levels of the α isozyme of PKC were normal in the prefrontal cortex of unipolar (medicated and unmedicated) depressed patients (Pacheco et al. 1996) and in the frontal cortex, amygdala, substantia nigra and putamen of unmedicated unipolar depressed patients (Hrdina et al. 1998). Decreased PKC activity was found in Brodmann's Areas 8 and 9 from brains of suicide victims (Pandey et al. 1997). PKC levels in post-mortem schizophrenic brains were also decreased in the parahippocampal gyrus (Dean et al. 1997) but were normal in the striatum (Opeskin et al. 1996) compared with healthy controls. These studies suggest that PKC may be dysregulated in psychiatric disorders, and that the cause and direction of this change is likely different for each disorder. With regard to bipolar disorder, it is unclear from these studies whether these effects are a trait of the disorder or an effect of medication, but these preliminary findings do suggest a potential role for PKC in bipolar disorder.

Peripheral blood cell measures give stronger support to the post-mortem bipolar patient studies which suggest that PKC activity varies with mood state (Table 4), and to the animal studies which suggest that PKC activity may be attenuated by mood stabilizers (Table 2). In platelets from bipolar patients in the manic state, the ratios of membrane PKC (active form) to cytosolic PKC (inactive form) were significantly elevated at baseline and following 5-HT-stimulation (Friedman et al. 1993, Wang et al. 1999) compared to healthy controls. Following two weeks of lithium treatment in manic bipolar patients, both membrane and cytosolic PKC levels were reduced to levels within the range found in healthy controls, and at this time 5-HT stimulation had no effect on the PKC ratio (Friedman et al. 1993). Another study reported that in euthymic patients taking lithium, PKC platelet cytosolic isozyme levels were reduced compared to controls (Soares et al. 1998). Recently, however, one study reported no differences in the α isozyme of PKC in the platelet membrane or cytosol, or in the membrane to cytosol ratio, in unmedicated bipolar patients or in lithium-treated bipolar patients compared to control subjects (Young et al. 1999). In contrast, unmedicated unipolar depressed patients had a higher level of platelet cytosolic PKC compared to controls, which may indicate a greater pool of inactive PKC in depressed patients (Pandey et al. 1998). This finding is supported by a recent finding that both thrombin or PMA (a direct activator of PKC) resulted in a low membrane-to-cytosolic ratio of PKC in platelets from unipolar depressed patients, as well as from schizophrenics (Wang et al. 1999).

Together, the clinical findings suggest that the levels of the active form of PKC may be increased in mania, following an abnormally enhanced activation of the PI-cycle. Depression, and possibly schizophrenia, may be associated with higher levels of the inactive (cytosolic) form of PKC, which is associated with a reduced response of the PI-cycle. These studies are consistent with the animal literature reporting attenuation of PKC activity by mood stabilizers. The therapeutic relevance of these findings has recently been tested in a small study, which described that tamoxifen, a PKC inhibitor, effectively stabilized mood in acutely manic patients (Manji et al. 1999). Peripheral cellular PKC activity is thus a potential marker of mood-state and may indicate a promising CNS target of future drug therapy for mood disorders.

Region	Diagnosis	Medication	Major Findings	Reference
Cortex, *post-mortem*	Bipolar	various mood stabilizers	↑ PKC α (S) ↑ PKC γ and ζ (P) ↑ PMA-induced redistribution of PKC (S) to PKC (P)	Wang and Friedman 1996
Platelets	Bipolar	No	N PKC α N PKCα (P:S ratio)	Young et al. 1999
Platelets	Bipolar	lithium	N PKC α N PKCα (P:S ratio)	Young et al. 1999
Platelets	Bipolar euthymia	lithium	↓ PKC α, β (I/II), δ, ε (S) N PKC α, β (I/II), δ, ε (P)	Soares et al. 1998
Platelets	Bipolar mania	no	↑ 5-HT-induced PKC (P:S ratio)	Friedman et al. 1996, Wang et al. 1999
Platelets	Bipolar mania	lithium (2 weeks)	N 5-HT-induced PKC (P:S ratio)	Friedman et al. 1993
Prefrontal cortex, *post-mortem*	Unipolar	various medications	N PKC α	Pacheco et al. 1996
Frontal cortex, amygdala, substantia nigra, putamen, *post-mortem*	Unipolar	no	N PKC α (P and S) ↓ GAP 43 (P)	Hrdina et al. 1998
Platelets	Unipolar	no	↑ PKC (S)	Pandey et al. 1998
Platelets	Unipolar depression	no	↓ thrombin/PMA-induced PKC (P:S ratio)	Wang et al. 1999

Table 4 - Protein Kinase C (PKC) Activity and Related Substrates in Affective Disorders

S soluble or cytosolic fraction; P particulate or membrane fraction; PMA phorbal 12-myristate 13-acetate; 5-HT serotonin; GAP-43 growth associated protein; ↑/↓/N increased, decreased or normal values compared to healthy controls.

1.6 Intracellular Ca^{2+} and the PI-Cycle

An important result of stimulation of receptors linked to the PI-cycle is an increase in intracellular Ca^{2+} (Berridge 1993). This is mainly due to the formation of IP_3 from the hydrolysis of PIP_2 by PLC. IP_3 diffuses through the cytosol and binds to and opens IP_3 receptors (ligand-gated calcium channels) on the endoplasmic reticulum and Ca^{2+} channels in the plasma membrane, causing Ca^{2+} to flow down its concentration gradient into the cytosol (Figure 2). In addition to regulation by the PI-cycle, intracellular Ca^{2+} levels are controlled within the cell by agonist- and voltage-gated Ca^{2+} channels on the plasma membrane (Kostyuk and Verkhratsky 1994). Since many cellular processes such as protein production, gene transcription, ionic distribution, cellular excitability and neurotransmitter release are dependent on intracellular Ca^{2+}, the regulation of intracellular Ca^{2+} concentrations is crucial for normal cellular functioning (Kostyuk and Verkhratsky 1994).

Altered cellular Ca^{2+} homeostasis, which often results in an abnormal increase in intracellular Ca^{2+} levels, is implicated in several pathological states (Berridge 1994, Mooren and Kinne 1998), including psychiatric disorders (Dubovsky and Franks 1983, Berridge 1994, Helmeste and Tang 1998). A hyperactivity in pathways that stimulate an increase in intracellular Ca^{2+} levels may be implicated in these Ca^{2+} abnormalities. Thus abnormal PI-cycle signaling, as reported in bipolar disorder, may ultimately alter Ca^{2+}-dependent cellular activity and communication, and manifest in the clinical symptoms seen in bipolar disorder. In support of the role of Ca^{2+} in bipolar disorder is evidence that (i) mood stabilizers attenuate intracellular Ca^{2+} influx in rat brain and (ii) peripheral blood cell Ca^{2+} influx is abnormal in bipolar disorder.

1.6.1 Mood Stabilizers Block Ca^{2+} Currents

There is evidence that the anticonvulsant mood stabilizers directly inhibit Ca^{2+} channels and as a result reduce Ca^{2+} currents in rat neuronal cells. Valproic acid reduced Ca^{2+} currents in ganglia (Kelly et al. 1990, Todorovic and Lingle 1998), hippocampal slices (Agopyan et al. 1985, Heinemann et al. 1985, Franceschetti et al. 1986), and at high concentrations in human neocortical cells (Sayer et al. 1993). Carbamazepine decreased Ca^{2+} currents in snail ganglia (Winkel and Lux 1987), rat ganglia and hippocampal neurons (Walden et al. 1992, Schirrmacher et al. 1993), and potentiated verapamil- (a Ca^{2+} channel blocker) induced reduction in Ca^{2+} currents in models of epilepsy (Walden et al. 1993). Lamotrigine reduced Ca^{2+} currents in amygdala neurons (Wang et al. 1996, 1998). Lithium reduced Ca^{2+} influx in response to stimuli in neurons, but this may have been an indirect consequence of a decreased release of glutamate (Khodorov et al. 1999), since lithium does not appear to bind directly to Ca^{2+} channels (Silverstone and Grahame-Smith 1991). Reduction of neuronal Ca^{2+} currents by mood stabilizers may lead to a reduction in cellular Ca^{2+} availability and a subsequent decrease in cellular firing and neurotransmitter release. This may be a mechanism by which these drugs attenuate cellular signaling pathways, resulting ultimately in a decreased activation of the PI-cycle in response to agonists or electrical stimulation, an effect which could potentially contribute to their mood stabilizing properties in bipolar patients. These suggestions are further supported by the therapeutic symptom reduction reported in bipolar patients in response to adjunctive or monotherapy with Ca^{2+} channel blockers (Giannini et al. 1985, Brunet et al. 1990, Garza-Trevino et al. 1992, Lindelius and Nilsson 1992, Dubovsky 1993, Pazzaglia et al. 1993, Walden et al. 1995, Hollister and

Trevino 1999).

1.6.2 Measurement of Intracellular Ca^{2+} Using Ratiometic Fluorescent Probes

Fluorescent Ca^{2+} chelators have been developed to measure free intracellular nanomolar (nM) Ca^{2+} levels in intact cells (Rink et al. 1982, Grynkiewicz et al. 1985, Rao et al. 1985, Rink et al. 1988, Iredale and Dickenson 1995). These indicators have the basic structure of the Ca^{2+} chelators ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA) and 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid BAPTA (Figure 5). These indicators can absorb and emit ultraviolet (UV) light, which allows their detection and quantification. Acetoxymethyl (AM) ester groups facilitate cellular loading, since the hydrophobic nature of these groups allows the compound to easily cross the cell membrane. Once inside, cellular esterases can cleave off this hydrophobic group and leave the charged form of the indicator "trapped" inside the cell.

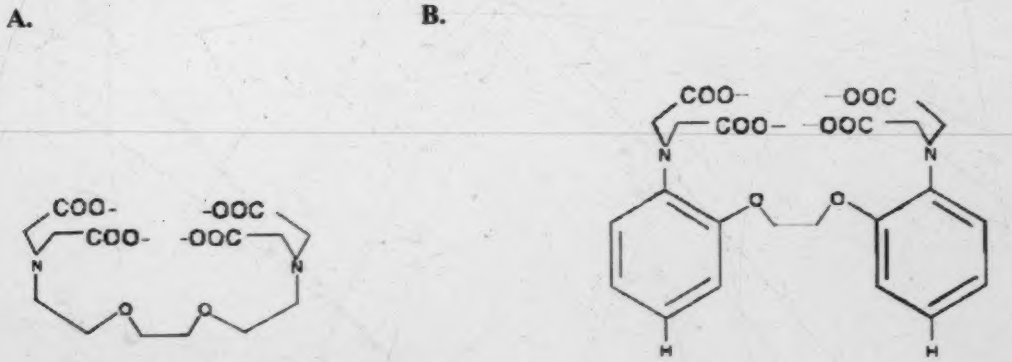

Figure 5 - Structure of Ca^{2+} Chelators
EGTA (A) and BAPTA (B).

There are two main types of fluorescence indicators: single wavelength and dual wavelength indicators (Grynkiewicz et al. 1985, Rao et al. 1985, Rink et al. 1988). Single wavelength indicators absorb UV light at a single wavelength in the unbound form and when bound to Ca^{2+}, giving one spectral signal. Dual-wavelength or ratiometric indicators have recently been developed, which shift absorbance wavelengths upon binding Ca^{2+}, and thus produce two spectral signals representing the bound and unbound form of indicator (Owen 1991). Fura-2 is an example of a dual-excitation indicator and is illustrated in Figure 7 below.

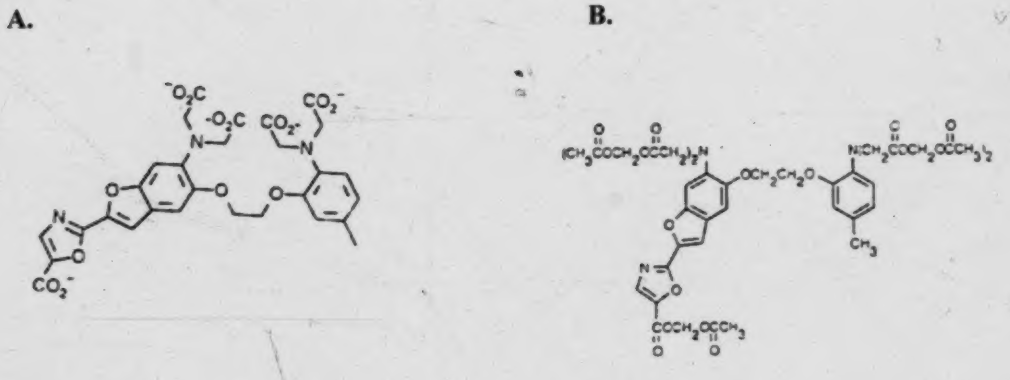
A.
B.
⁻O₂C
-O₂C
CO₂-
CO₂⁻
N
O
CO₂⁻
(CH₃COCH₂OCCH₂)₂N
OCH₂CH₂O
N(CH₂COCH₂OCCH₃)₂
CH₃
COCH₂OCCH₃

Figure 6 - Structure of Fura-2
Fura-2 Anion (A) and Fura-2AM (B).

With all fluorescent indicators, free intracellular Ca^{2+} concentrations are quantified by performing end-calibrations in the presence of minimum (zero Ca^{2+}) and maximum (excess Ca^{2+}) concentrations, usually by lysing the cells in the presence of EGTA or adding millimolar quantities of Ca^{2+}, respectively. The derivation of formulas for both single and dual-excitation indicators has facilitated the determination of Ca^{2+} concentrations using the fluorescence signals obtained during measurements and calibrations (Grynkiewicz et al 1985):

Equation (1)

$$[Ca^{2+}] = K_d\,[(F - F_{min})/(F_{max} - F)]$$

(F = fluorescent intensity, fluorescence, F_{min} = F at minimal $[Ca^{2+}]$, F_{max} = F at maximal $[Ca^{2+}]$)

Equation (2)

$$[Ca^{2+}] = K_d\,[(R - R_{min})/\,(R_{max} - R)](S_{f2} / S_{b2})$$

(R (ratio of fluorescent signals) = $F_{\lambda 1}$ / $F_{\lambda 2}$, R_{min} = R in the presence of minimal $[Ca^{2+}]$, R_{max} = R in the presence of maximal $[Ca^{2+}]$, S_{f2} = fluorescence of free indicator at λ2 (i.e. minimal $[Ca^{2+}]$), F_{b2} = fluorescence of bound indicator at λ2 (i.e. maximal $[Ca^{2+}]$)

The approximate dissociation constant (K_d) of Fura-2 is 224 nM as measured in cytosolic-like buffers at 37°C (Grykiewicz et al. 1985). This K_d is ideal for the measurement of platelet Ca^{2+} concentrations, which range from 50 to 250 nM under basal conditions (Rink et al. 1982, Rink and Hallam 1984). While the absorbance wavelength of Fura-2 can shift from 0.1 x K_d to 10 x K_d, the most accurate measurements are obtained when the Ca^{2+} concentrations are about equal to or slightly below the K_d (Meyer et al. 1990, Takahashi et al. 1999). In general, ratiometric determinations give more accurate measures of Ca^{2+} concentrations than single wavelength indicators because the use of ratios reduces errors due to autofluorescence, indicator leakage and uneven dye loading between batches of cells (Grykiewicz et al. 1985). A number of studies, reviewed below, have used ratiometric indicators to measure peripheral blood cell Ca^{2+} concentrations from bipolar patients.

1.6.3 Enhanced Ca^{2+} Response in Peripheral Blood Cells of Bipolar Patients

There are reports of abnormally elevated *basal* levels of intracellular Ca^{2+} in blood cells from bipolar patients (Table 5). Elevated basal Ca^{2+} levels have been found in platelets from unmedicated bipolar patients in the manic state (Dubovsky et al. 1989) and in the depressed state (Dubovsky et al. 1991), and in euthymic bipolar patients treated with lithium (Tan et al. 1990, Berk et al. 1994, 1995). Studies using groups of bipolar patients in various states report elevated platelet and lymphocyte Ca^{2+} (Dubovsky et al. 1992, Hough et al. 1999) and elevated B-lymphoblast Ca^{2+} (Emamghoreishi et al. 1997). In contrast, normal blood cell levels of Ca^{2+} are reported in symptomatic unipolar depressed (Dubovsky et al. 1989, 1991, Kusumi et al. 1992, Mikuni et al. 1992, Kusumi et al. 1994b, Yamawaki et al. 1996, Akiyoshi et al. 1997, Emamghoreishi et al. 1997, Delisi et al. 1998) and non-symptomatic patients (Bothwell et al. 1994, Berk et al. 1998, Tomiyoshi et al. 1999), with the exception of one study which reported an increase in platelet Ca^{2+} in unipolar depressed male patients (Konopka et al. 1996). However, there are several reports of normal cellular Ca^{2+} levels in bipolar mania (Berk et al. 1994, 1995, Okamoto et al. 1995, Tan et al. 1995), bipolar depression (Dubovsky et al. 1989, Kusumi et al. 1992, 1994b, Berk et al. 1994, 1995), and in lithium-treated bipolar patients (Dubovsky et al. 1989, 1991, Bothwell et al. 1994, Okamoto et al. 1995). Thus, basal platelet Ca^{2+} concentrations may be normal in euthymia, and increase with depression and mania.

Subject & State	n	$[Ca^{2+}]_i$ (nM)	Compared to HC	Cells	Psychotropic Medication	References
BP – manic	21	48	N	platelets	no	Berk et al. 1994, 1995
BP – manic	10	82	N	platelets	no	Okamoto et al. 1995
BP – manic	7	127	N	platelets	haloperidol	Tan et al. 1995
BP – manic	15	157	↑	platelets	no	Dubovsky et al. 1989
BP – depressed	16	72	N	platelets	no	Kusumi et al. 1994b
BP – depressed	19	49	N	platelets	no	Berk et al. 1994, 1995
BP – depressed	14	73	N	platelets	no	Kusumi et al. 1992
BP – depressed	15	136	↑ns	platelets	no	Dubovsky et al. 1989
BP – depressed	15	231	↑	platelets	no	Dubovsky et al. 1991
BP – euthymic	6	320	↑	platelets	lithium	Tan et al. 1990
BP – euthymic	20	51	↑	platelets	lithium	Berk et al. 1994, 1995]
BP – euthymic	10	75	N	platelets	Li + various	Okamoto et al. 1995
BP – euthymic	17	88	N	platelets	Li + various	Bothwell et al. 1994
BP – euthymic	13	112	N	platelets	Li + various	Dubovsky et al. 1989
BP – euthymic	9	143	N	platelets	Li + various	Dubovsky et al. 1991
BP – man/dep	13	82	N	platelets	no	Yamawaki et al. 1996
BP – man/dep	9	121	↑	platelets	no	Dubovsky et al. 1992
BP – man/dep	9	129	↑	lymphocytes	no	Dubovsky et al. 1992
BP(I) – man/dep	11/10	78/105	N	T-lymphocytes	various	Emamghoreishi et al. 1997
BP(I) – man/dep	14/14	64/63	↑	B-lymphoblasts	various	Emamghoreishi et al. 1997
BP(II) – man/dep	2/6	69/72	N	T-lymphocytes	various	Emamghoreishi et al. 1997
BP(II) – man/dep	5/5	61/57	N	B-lymphoblasts	various	Emamghoreishi et al. 1997
BP – unspecified	24	87	↑	lymphocytes	various	Hough et al. 1999
BP – unspecified	39	153	↑	platelets	various	Hough et al. 1999
BP/UP – depressed	10	104	N	platelets	antidepressant	Eckert et al. 1993
BP/UP – depressed	10	104	N	platelets	antidepressant	Eckert et al. 1994
BP/UP – depressed	10	137	N	lymphocytes	antidepressant	Eckert et al. 1994
HT – depressed	23	51/61	N	platelets	AH	Delisi et al. 1999

UP – depressed	24	62/64	N	platelets	various	Delisi et al. 1998
UP – depressed	7/5	84/72	N	B-lymphoblasts	various	Emamghoreishi et al. 1997
UP – depressed	11	69	N	platelets	no	Mikuni et al. 1992
UP – depressed	10/9	84/106*	↑/↑*	platelets	various	Konopka et al. 1996
UP – depressed	26	68	N	platelets	no	Kusumi et al. 1994b
UP – depressed	8/4	57/58	N	B-lymphoblasts	various	Emamghoreishi et al. 1997
UP – depressed	7/5	84/72	N	T-lymphocytes	various	Emamghoreishi et al. 1997
UP – depressed	23	68	N	platelets	no	Kusumi et al. 1992
UP – depressed	12	78	N	platelets	no	Yamawaki et al. 1996
UP – depressed	13	124	N	platelets	no	Dubovsky et al. 1989
UP – depressed	12	98	N	platelets	no	Akiyoshi et al. 1997
UP – depressed	9	158	N	platelets	no	Dubovsky et al. 1991
UP - depressed	21	111	N	MNL	AD/AP	Adunsky et al. 1995
BP/UP – euthymic	14	nr	N	neutrophils	lithium + AD	Forstner et al. 1994
BP/UP – remission	14	89	N	platelets	lithium	Bothwell et al. 1994
UP – remission	18	86	N	platelets	imipramine	Tomiyoshi et al. 1999
UP – remission	27	83	N	platelets	Li + various	Bothwell et al. 1994
UP – remission	24	83	N	platelets	no	Tomiyoshi et al. 1999
UP – subsyndromal	16	103	↑ns	platelets	no	Berk et al. 1998
UP – unspecified	12	80	N	platelets	various	Hough et al. 1999
UP – unspecified	9	83	N	lymphocytes	various	Hough et al. 1999
HT – euthymic	25	54/64	N	platelets	AH	Delisi et al. 1999

Table 5 - Basal Peripheral Blood Cellular Ca^{2+} Concentrations in Affective Disorders

$[Ca^{2+}]_i$ intracellular Ca^{2+} concentration; nM nanomoles per litre; BP bipolar disorder; I bipolar I disorder; II bipolar II disorder; UP unipolar disorder (major depressive disorder); HT hypertensive patients; man manic; dep depressed; ↑/↓/N increased, decreased or normal values relative to healthy controls; ns not statistically significant; Li lithium; various includes mood stabilizers, antidepressants or antipsychotics; AH antihypertensive agents; AD antidepressant; AP antipsychotics; MNL mononuclear leukocytes; * first value obtained in absence of extracellular Ca^{2+} and second value in the presence of 1 mM extracellular Ca^{2+}; nr not reported.

More consistently, an enhanced Ca^{2+} *response* (increase or rise in cytosolic Ca^{2+}) to agonist stimulation in bipolar disorder has been reported (Table 6). An enhanced Ca^{2+} response was found in platelets from unmedicated bipolar patients in the manic state in response to 5-HT (Berk et al. 1995, Okamoto et al. 1995, Yamawaki et al. 1996) and thrombin (Dubovsky et al. 1989, Tan et al. 1995) and in the depressed state in response to 5-HT (Kusumi et al. 1994b, Berk et al. 1995) and thrombin (Dubovsky et al. 1989, 1991, Kusumi et al. 1992). In bipolar patients in various mood states, taking various medications, an enhanced Ca^{2+} response has also been found in platelets in response to 5-HT (Yamawaki et al. 1996, Hough et al. 1999) and to thrombin and thapsigargin (a Ca^{2+}-ATPase inhibitor which blocks Ca^{2+} repletion of the ER) (Hough et al. 1999). These peripheral cell findings indicate that an overactive PI-cycle, assessed by an enhanced Ca^{2+} response to agonists linked to the PI-cycle, may exist in bipolar disorder and may be related to clinical symptoms and mood state. This idea is supported by the findings that the Ca^{2+} response to 5-HT (Bothwell et al. 1994, Berk et al. 1995, Okamoto et al. 1995) and thrombin (Dubovsky et al. 1989, 1991, Bothwell et al. 1994) in platelets of euthymic bipolar patients taking lithium, was found to be normal. Thus, it is possible that this enhanced response may either normalize in euthymia or with lithium medication.

In comparison, unipolar depressive disorder may also be characterized by similar abnormalities in peripheral blood cell Ca^{2+} responsiveness. An enhanced 5-HT Ca^{2+} response was found in unipolar depressed patients (Mikuni et al. 1992, Kusumi et al. 1994b, Konopka et al. 1996, Yamawaki et al. 1996, 1998, Delisi et al. 1998) and in euthymic patients diagnosed with unipolar depression (Tomiyoshi et al. 1999). This may be the result of a specific abnormality in 5-HT receptor-linked PI hydrolysis in both unipolar and bipolar affective disorders. In contrast to bipolar disorder, peripheral blood cells from unipolar patients exhibited a normal thrombin-induced Ca^{2+} response in the depressed state (Dubovsky et al. 1989, 1991, Kusumi et al. 1992) and in the euthymic state (Berk et al. 1998, Bothwell et al. 1994, Hough et al. 1999, Tomiyoshi et al. 1999). These findings suggest that subtypes of depression with different pathophysiological profiles may have corresponding differences in platelet Ca^{2+} responsiveness to further support evidence indicating that unipolar and bipolar depression may have different etiologies.

An enhanced Ca^{2+} response to thrombin was also reported in peripheral blood cells in other psychiatric disorders including panic disorder (Plein and Berk 1999) and schizophrenia (Das et al. 1995, Konopka et al. 1996). However, a normal response to 5-HT has also been reported in panic disorder (Plein and Berk 1999) and schizophrenia (Tan et al. 1995, Ereshefsky et al. 1996, Konopka et al. 1996). These results suggest that although there may be a similar enhancement in Ca^{2+} response in other psychiatric disorders under some circumstances, the underlying mechanism of this response may be different from that measured in bipolar disorder.

To isolate the effects of mood stabilizers on the proposed enhancement of Ca^{2+} response in peripheral cells in bipolar disorder, some investigators have incubated mood stabilizers with peripheral blood cells. Lithium inhibited basal $^{45}Ca^{2+}$ uptake into platelets from both manic and depressed bipolar patients, as well as from controls (Berk et al. 1996). *In vitro* incubation of lymphocytes with carbamazepine significantly attenuated both the basal and PHG-stimulated Ca^{2+} rise in manic and depressed bipolar patients, but not in healthy controls (Dubovsky et al. 1994). However, acute *in vitro* incubation of lithium, carbamazepine or valproic acid with platelets, and chronic treatment with lithium, did not alter 5-HT or thrombin-induced Ca^{2+} responses in healthy controls (Kusumi et al 1994a). Flunarazine and verapamil, two calcium channel blockers, were found to induce a greater inhibition of basal $^{45}Ca^{2+}$ uptake into platelets from manic bipolar patients relative to controls (Berk and Kirchmann 1995). These results provide further insight into the mechanisms by which mood stabilizers may attenuate cellular Ca^{2+} responses and give support to suggestions that overactive Ca^{2+} channels may be targets for mood stabilizers in symptomatic bipolar patients.

Subject & State	n	Agonist	$[Ca^{2+}]_i$ (nM) (PA)	Other Measures of $[Ca]^{2+}$ (nM)	Compared to HC	Cells	Psychotropic Medication	Reference
BP – manic	21	5-HT	159	-	↑ (vs euth)	platelets	no	Berk et al. 1995
BP – manic	10	5-HT	-	83 (Δ)	↑	platelets	no	Okamoto et al. 1995
BP – manic	21	dopamine	~ 80	-	N	platelets	no	Berk et al. 1994
BP – manic	13	thrombin & PAF	855	-	↑	platelets	no	Dubovsky et al. 1989
BP – manic	nr	5-HT	-	83 (Δ)	↑	platelets	nr	Yamawaki et al. 1998
BP – manic	7	thrombin	-	309 (Δ)	↑ns	platelets	haloperidol	Tan et al. 1995
BP – depressed	19	5-HT	141	-	↑ (vs euth)	platelets	no	Berk et al. 1995
BP – depressed	19	dopamine	~ 75	-	N	platelets	no	Berk et al. 1994
BP – depressed	14	thrombin	454	-	↑	platelets	no	Dubovsky et al. 1991
BP – depressed	14	thrombin	-	1984 (% basal)	↑	platelets	no	Kusumi et al. 1992
BP – depressed	14	thrombin & PAF	769	-	↑	platelets	no	Dubovsky et al. 1989
BP – depressed	16	5-HT	-	123 (Δ)	↑	platelets	no	Kusumi et al. 1994b
BP – euthymic	9	fMLP	-	~ 2.5 (EC_{50})	↑	neutrophils	lithium	van Calker et al. 1993
BP – euthymic	6	thrombin	-	368 (Δ)	↑	platelets	lithium	Tan et al. 1990

BP – euthymic	17	5-HT	120		N	platelets	Li + various	Bothwell et al. 1994
BP – euthymic	10	5-HT	-	61 (Δ)	N	platelets	Li + various	Okamoto et al. 1995
BP – euthymic	17	PAF	197		N	platelets	Li + various	Bothwell et al. 1994
BP – euthymic	17	thrombin	393		N	platelets	Li + various	Bothwell et al. 1994
BP – euthymic	8	thrombin	349	-	N	platelets	Li + various	Dubovsky et al. 1991
BP – euthymic	12	thrombin & PAF	513	-	N	platelets	Li + various	Dubovsky et al. 1989
BP – euthymic	20	5-HT	96	-	N	platelets	lithium	Berk et al. 1995
BP – euthymic	20	dopamine	~ 70	-	N	platelets	lithium	Berk et al. 1994
BP – man/dep	13	5-HT	-	83 (Δ)	↑	platelets	no	Yamawaki et al. 1996
BP(I) – man/dep	11/ 10	PHG	150/195 +	-	N/N	T-lymphocytes	various	Emamghoreishi et al. 1997
BP(II) – man/dep	2/6	PHG	162/146 +	-	N/N	T-lymphocytes	various	Emamghoreishi et al. 1997
BP – unspecified	24	conA/PMA	-	13 (Δ)	N	lymphocytes	various	Hough et al. 1999
BP – unspecified	34	5-HT	-	16 (Δ)	↑	platelets	various	Hough et al. 1999
BP – unspecified	26	5-HT	-	6 (Δ)	N	lymphocytes	various	Hough et al. 1999

BP – unspecified	36	NaF	-	20 (Δ)	N	platelets	various	Hough et al. 1999
BP – unspecified	23	NaF	-	7 (Δ)	N	lymphocytes	various	Hough et al. 1999
BP – unspecified	24	thapsigargin	-	564 (Δ)	↑	platelets	various	Hough et al. 1999
BP – unspecified	21	thapsigargin	-	394 (Δ)	↑	lymphocytes	various	Hough et al. 1999
BP – unspecified	37	thrombin	-	18 (Δ)	↑	platelets	various	Hough et al. 1999
BP/UP – depressed	10	5-HT	262	159 (Δ)	↑/↑	platelets	antidepressant	Eckert et al. 1993
BP/UP – depressed	10	5-HT	-	159 (Δ)	↑	platelets	antidepressant	Eckert et al. 1994
BP/UP – depressed	10	PHA	-	65 (Δ)	↓	lymphocytes	antidepressant	Eckert et al. 1994
BP/UP – depressed	10	thrombin	-	840 (Δ)	N	platelets	antidepressant	Eckert et al. 1994
BP/UP – depressed	10	thrombin	976	-	N	platelets	antidepressant	Eckert et al. 1993
BP/UP – depressed	13	5-HT	-	267 (% basal)	↑	platelets	no	Kusumi et al. 1991
BP/UP – depressed	17	fMLP	-	1.55 (EC_{50}) 165 (Δ)	↓ ↑	neutrophils	no	Bohus et al. 1996
BP/UP – depressed	13	thrombin	-	1773 (% basal)	N	platelets	no	Kusumi et al. 1991
BP/UP – dep/euth	14	fMLP	-	~1.5 (EC_{50})	↓	neutrophils	no	van Calker et al. 1993

UP – depressed	26	5-HT	-	114 (Δ)	↑	platelets	no	Kusumi et al. 1994b
UP – depressed	nr	5-HT	-	83 (Δ)	↑	platelets	nr	Yamawaki et al. 1998
UP – depressed	11	5-HT	-	33/77* (Δ)	↑ (vs UP non-SSRI)	platelets	SSRI	Delisi et al. 1998
UP – depressed	21	PHA	1071	960 (Δ)	↑/↑	MNL	AD/AP	Adunsky et al. 1995
UP – depressed	12	5-HT	-	83 (Δ)	↑	platelets	no	Yamawaki et al. 1996
UP – depressed	11	5-HT	-	129 (Δ)	↑	platelets	no	Mikuni et al. 1992
UP – depressed	12	CCK-4	162	-	N	T-cells	no	Akiyoshi et al. 1997
UP – depressed	11	NE	-	46 (Δ)	N	platelets	no	Mikuni et al. 1992
UP – depressed	9	thrombin	362	-	N	platelets	no	Dubovsky et al. 1991
UP – depressed	23	thrombin	-	1486 (% basal)	N	platelets	no	Kusumi et al. 1992
UP – depressed	12	thrombin & PAF	388	-	N	platelets	no	Dubovsky et al. 1989
UP – depressed	24	5-HT	103/172*	41/109* (Δ)	N/↑; ↑/↑	platelets	various	Delisi et al. 1998
UP – depressed	10/9	5-HT	98/149*	-	↑/↑	platelets	various	Konopka et al. 1996
UP – depressed	7/5	PHG	153/145$^+$	-	N/N	T-lymphocytes	various	Emamghoreishi et al. 1997
HT – depressed	12	5-HT	-	29/83 (Δ)	N/↑ (vs HT-euth)	platelets	AH	Delisi et al. 1999

BP/UP – euthymic	14	5-HT	123		↑	platelets	lithium	Bothwell et al. 1994
BP/UP – euthymic	14	PAF	192		N	platelets	lithium	Bothwell et al. 1994
BP/UP – euthymic	14	thrombin	390		N	platelets	lithium	Bothwell et al. 1994
BP/UP – euthymic	14	fMLP	-	~225 (Δ)	↓	neutrophils	lithium +AD	Forstner et al. 1994
HT – euthymic	25	5-HT	-	25/60 (Δ)	N/N	platelets	AH	Delisi et al. 1999
UP – remission	18	5-HT	-	119 (Δ)	↑	platelets	imipramine	Tomiyoshi et al. 1999
UP – remission	27	5-HT	109		N	platelets	Li + various	Bothwell et al. 1994
UP – remission	27	PAF	183		N	platelets	Li + various	Bothwell et al. 1994
UP – remission	27	thrombin	431		N	platelets	Li + various	Bothwell et al. 1994
UP – remission	24	5-HT	-	106 (Δ)	↑	platelets	no	Tomiyoshi et al. 1999
UP – subsyndromal	16	5-HT	138	-	↑ns	platelets	no	Berk et al. 1998
UP – subsyndromal	16	thrombin	240	-	↑ns	platelets	no	Berk et al. 1998
UP – unspecified	12	5-HT	-	14 (Δ)	N	platelets	various	Hough et al. 1999
UP – unspecified	8	5-HT	-	6 (Δ)	N	lymphocytes	various	Hough et al. 1999
UP – unspecified	8	conA/PMA	-	16 (Δ)	N	lymphocytes	various	Hough et al. 1999

UP – unspecified	14	NaF	-	36 (Δ)	N	platelets	various	Hough et al. 1999
UP – unspecified	7	NaF	-	5 (Δ)	N	lymphocytes	various	Hough et al. 1999
UP – unspecified	5	thapsigargin	-	283 (Δ)	N	platelets	various	Hough et al. 1999
UP – unspecified	6	thapsigargin	-	488 (Δ)	N	lymphocytes	various	Hough et al. 1999
UP – unspecified	14	thrombin	-	17 (Δ)	N	platelets	various	Hough et al. 1999

Table 6 - Agonist-Induced Rises in Peripheral Blood Cellular Ca^{2+} Concentrations in Affective Disorders

$[Ca^{2+}]_i$ intracellular calcium concentration; nM nanomoles per litre; PA peak amplitude of calcium rise; Δ change in $[Ca^{2+}]_i$ relative to baseline; EC_{50} half-maximal effective concentration; HC healthy controls; BP bipolar disorder; I bipolar (I) disorder; II bipolar (II) disorder; UP unipolar disorder; HT hypertensive patients; dep depressed; euth euthymic; man manic; no medications indicates medication-free for at least one week; 5-HT serotonin; NaF sodium fluoride; PAF platelet-activating factor; PHG phytohemagglutinin; fMLP chemotactic peptide formylmethionylleucylphenylalanine; CCK cholecystokinin; NE norepinephrine; con A concanavalin A; PMA phorbol 12-myristate 13-acetate; Li lithium; various includes mood stabilizers, antidepressants or antipsychotics; SSRI selective serotonin reuptake inhibitors; AP antipsychotics; AH antihypertensive agents; MNL mononuclear leukocytes; AD antidepressant; ↑/↓N increased, decreased or normal concentrations compared to controls unless otherwise stated; ns not significantly different from controls; * first value obtained in absence of extracellular calcium and second value in the presence of 1 mM extracellular calcium; + first value females only and second value males only; nr not reported; - measure not reported.

1.7 Summary of Research on the Inositol-Depletion Hypothesis

Although there is abundance of evidence suggesting the PI-cycle is abnormal in bipolar patients, and is altered by mood stabilizers, the animal and human data are not fully consistent with the inositol-depletion hypothesis. Indeed, while an increase in I-1-P and a decrease in *myo*-inositol in response to lithium is predicted from this hypothesis, both supportive and opposing findings have been reported in animals. In human studies, opposing data are frequently found, such as a decrease in PME levels often reported in medicated bipolar patients. Human studies should be interpreted with caution as data may be confounded by trait- or state-specific phenomena of bipolar disorder, and the heterogeneous use of medication. In general, however, the collection of animal and patient literature to date supports the suggestions that (1) a PI-cycle abnormality is often associated with bipolar disorder and (2) mood stabilizers alter PI-cycle components in both resting and activated cells. To help determine the implications and precise mechanisms of these effects, it is useful to examine changes found in other components of the PI-cycle which are closely linked to the metabolism of I-1-P to *myo*-inositol, such as membrane phosphoinositides and phospholipids, G-proteins, PKC and intracellular Ca^{2+}. These components may be more closely associated with the manifestation of the clinical symptoms and the therapeutic effects of chronic medication use in bipolar disorder.

1.8 Rationale and Hypothesis

The objective of this thesis is to further investigate the PI-cycle activation of increased cytolsolic Ca^{2+} concentration in bipolar disorder. Evidence for involvement of intracellular Ca^{2+} in bipolar disorder includes (i) reports of abnormal agonist-induced changes in platelet Ca^{2+} concentrations found in bipolar patients, (ii) evidence that mood stabilizers block Ca^{2+} channels in rat brain and (iii) clinical studies showing that Ca^{2+} channel blockers are useful therapeutic agents in bipolar patients. The Ca^{2+} hypothesis of bipolar disorder, which arises from the literature, suggests that (i) an abnormality in intracellular Ca^{2+} homeostasis is present in bipolar disorder and results in hypersensitive and overactive cellular signaling pathways and (ii) mood stabilizers exert their therapeutic effects by attenuating intracellular Ca^{2+} influx.

In vivo MRS data gathered in our laboratory support the involvement of the PI-cycle in bipolar disorder by showing an increase in PME, a measure of IP, in healthy subjects challenged with dextroamphetamine, but not in euthymic bipolar patients taking either chronic lithium or sodium valproate (Silverstone et al. 2000, unpublished data). These findings suggest that lithium and valproate attenuate agonist-induced PI-cycle activation.

Based on these findings and the Ca^{2+} hypothesis, the hypotheses addressed in this thesis state that: (i) dextroamphetamine, a psychological and pharmacological model of mania, will produce an enhanced thrombin- and 5-HT-induced increase in platelet Ca^{2+} concentrations in healthy male volunteers when administered *in vivo*, or when incubated with platelets *in vitro*, and (ii) the mood stabilizers lithium, sodium valproate and carbamazepine will attenuate thrombin- and 5-HT-induced increases in platelet Ca^{2+}

concentrations when incubated with cells *in vitro*. The overall goal of this project is to gain insight into the mechanisms of action of mood stabilizers, dextroamphetamine and mania. In preparation for this study, it was necessary to develop a fluorometric assay to measure platelet Ca^{2+} concentrations using the fluorescence indicator Fura-2.

CHAPTER 2 – METHODS

2.1 Introduction

A fluorometric assay to measure platelet intracellular Ca^{2+} concentration, $[Ca^{2+}]$ was modified from previously described methods (Rao et al. 1985, Dubovsky et al. 1989, 1991, 1992, Tan et al. 1990, 1995, Kusumi et al. 1991, 1992, Mikuni et al. 1992, Eckert et al. 1993, 1994, Berk et al. 1994, 1995, 1998, Okamoto et al. 1995, Sage 1996, Fowler et al 1997, van Gorp et al 1997, Siafaka-Kapadai et al 1997, Delisi et al. 1998, 1999, Yamawaki et al. 1998, Yoshimura et al 1998, Tomiyoshi et al. 1999). In this section the protocol used in this study (Appendix 2) for measurement of platelet $[Ca^{2+}]$ in healthy subjects using the fluorescence indicator Fura-2 is described. Experimental manipulations and trouble-shooting that were performed are discussed at each major step in the protocol, including platelet isolation, dye loading and fluorometry, explaining why this particular method was selected over alternates. The major steps in the protocol involve platelet isolation, dye loading and fluorometry. In the method development, two types of fluorescence recordings are made: excitation scans and dual excitation traces over time. Excitation scans record fluorescence intensity over a range of wavelengths from 300 to 420 nanometers (nm) and illustrate the amount of dye in the bound (peak ~340) and unbound form (peak ~380). Dual-excitation excitation scans display the fluorescence intensity of the bound form (340 nm) and unbound form (380 nm) of Fura-2 over time. These scans can then be converted into $[Ca^{2+}]$ after end calibrations are performed in the presence of minimal and maximal $[Ca^{2+}]$. Both types of scans were used in determining optimal experimental conditions for measurements and are referred to in the trouble-shooting sections. A list of chemicals used is found in Appendix 1.

2.2 Subjects

A full physical examination, detailed history and electrocardiogram (ECG) reading were provided for all subjects. Subjects were excluded if they had high blood pressure or abnormal ECG readings. They were all males with no current medical or psychiatric illness and no personal history of psychiatric or cardiovascular illness, and were all medication-free. All subjects were free from recreational drug-use and were non-smokers for at least one year. They all refrained from the use of salicylic acid (Aspirin®), ibuprofen (Advil®), acetaminophen (Tylenol®) and any other drugs affecting platelet aggregation for at least two weeks prior to the start of the study. Subjects were required to fast from midnight and were allowed to drink only water throughout the study. Informed consent was obtained from all subjects prior to participation in the study.

2.2.1 Drugs Affecting Measurements

Several commonly used pain and cold medications inhibit platelet activation, including preparations of non-steroidal anti-inflammatory drugs (NSAIDs), salicylic acid, acetaminophen, and ibuprofen (Weiss 1972), while smoking increases platelet activation and aggregation (Roy 1999). Subjects taking drugs used in blood or coagulation disorders (O'Reilly 1995) should also be excluded as they can affect the platelet response measured by interfering with various pathways in platelet activation.

There is evidence that both endogenous and synthetic estrogens and progestogens are weak activators of platelet aggregation (Kuhl 1996), and thus oral contraceptives should be avoided, and hormonal variations due to the menstrual cycle should be considered in female subjects. Platelet Ca^{2+} responses were shown to vary with the female menstrual cycle (Pedersen and Reichelt 1988). Further, the psychological effects of dextroamphetamine were found to be enhanced in the follicular phase of the menstrual cycle, relative to the luteal phase, an effect which was positively correlated with estrogen levels (Justice and de Wit 1999). For these reasons only male subjects were included in this study.

2.3 Study Design

A double-blind placebo-controlled cross-over study design was used. Subjects (total of n = 15) came in on two separate occasions three to five weeks apart and were administered either 25 mg dextroamphetamine or an equivalent volume of placebo (lactose powder). Capsules were administered in a random manner such that each subject had a 50% chance of receiving either capsule on day one, and then received the alternate capsule on day two. This dose of dextroamphetamine was chosen because, in a previous study, a 20 mg dose of dextroamphetamine was effective in inducing significantly increased psychological effects of happiness, alertness, energy, thought speed, light-headedness and restlessness, subjectively reported by healthy subjects on visual analogue scales (V.A.S.) 2 hours after administration (Silverstone et al. 2000, unpublished data).

2.4 Blood Draw and Platelet Isolation

Blood (45 mL) was drawn between the hours of 7:00 and 8:00 am, and then again 3.5 hours after administration of the capsules. Experiments were performed immediately after the blood draw, and lasted for approximately 3.5 hours. The blood was collected using a 19-gauge butterfly needle set attached to a 60cc polypropylene syringe containing

1:9 mL citrate buffer (3.15% sodium citrate). Blood was transferred into a polypropylene tube to which 0.06 μg/mL prostaglandin I_2 (prostacyclin, PGI_2) was added and centrifuged at 375 g for 20 minutes at room temperature. Platelet-rich plasma (PRP) (18-25 mL) was removed and 0.3 μg/mL PGI_2 was added. PRP was then centrifuged at 2240 g for 10 minutes at room temperature. Platelet-poor plasma (PPP) was removed and the platelet pellet was re-suspended in 3-5 mL HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer [10 mM HEPES, 137 mM NaCl, 1.8 mM $CaCl_2$ dihydrate, 1.0 mM $MgCl_2(6H_2O)$, 5.5 mM glucose, 2.7 mM KCl, 0.4 mM Na_2HPO_4 (dibasic), pH 7.3 with 1.0 M TRIS (2-(hydroxymethyl)aminomethane) buffer (0.67 M Trizma base, 0.30 M TRIS HCl, pH 9.0], containing 1:9 citrate buffer.

2.4.1 Avoiding Platelet Activation

Platelets are very fragile and easily activated once removed from their natural environment (Radomski et al. 1996). Activation is a normal receptor-mediated response to agonists or shear stress, which results in the initiation of signal transduction pathways such as the phosphoinositide and cyclic-AMP pathways (Scrutton 1993). Activation of these pathways in the platelet results in a cascade of events, in particular an increase in cytosolic [Ca^{2+}] and morphological changes, leading to platelet aggregation (Sage et al. 1993, Scrutton 1993). Aggregation is a phenomenon in which platelets stick together via the attachment of fibrinogen between receptors of the plasma membranes forming clots (Siess 1989). It is essential that platelets do not become activated to any degree at anytime during their use as this process is usually irreversible (Siess 1989). Activated platelets stick together and do not suspend evenly in solutions. Even partial activation of platelets can obscure measurements due to an abnormal elevation of basal Ca^{2+} concentrations, and functional responses in even slightly activated platelets will result in inaccurate measurements (Radomski, personal communication).

In addition to physiological agonists, platelets can be activated by a number of factors during handling procedures such as material surfaces, movement, temperature and platelet concentration (Radomski, personal communication). Glass induces platelet activation and so plastic, polypropylene, polyethylene or silconized glass containers are preferable (Mustard et al. 1989). Centrifuging and shaking apparati induce platelet activation, which can be avoided by the addition of an anticoagulant. Platelet suspensions are more easily activated when they are stored above room temperature, and when they are concentrated above normal physiological concentrations ($1.5 - 4.0 \times 10^8$ cells/mL) (Clemetson 1996, Poole 1996) and thus long-term exposure to these environments should be avoided where possible.

By implementing proper handling techniques and using appropriate anti-coagulants, platelet activation can usually be avoided. Citrate buffers are weak anticoagulants which act by chelating Ca^{2+} and thus inhibiting platelet activation due to cellular Ca^{2+} rises (McNicol 1996). These buffers are particularly useful when used in 1:9 ratios in the collection of whole blood, producing citrated platelet rich plasma after centrifugation. Commonly used solutions include 3.15% sodium citrate, 2-3% citrate-citric acid-dextrose (CCD) and 1.5-2.5% acid-citric acid-dextrose (ACD) (McNicol 1996). Stronger Ca^{2+} chelators, such as EDTA and EGTA are also effective, but they have the disadvantage of inhibiting normal cellular function by altering membrane receptors and depleting Ca^{2+} stores (McNicol 1996). Aspirin inhibits ADP production resulting in decreased platelet activation (Weiss 1972, Rao and White 1994). However, this effect can last up to 2 weeks and can thus affect functional measurements. Apyrase, an enzyme isolated from potatoes, also decreases ADP-induced platelet activation. This enzyme scavenges ADP and is a weak inhibitor of platelet activation (Sage 1996). PGI_2 is a potent inhibitor of platelet activation which acts by increasing cyclic AMP and inhibiting pathways involved in calcium influx (Radomski and Moncada 1983). PGI_2 has a short half-life above 0°C and thus allows quick recovery of normal platelet function (Radomski and Moncada 1983).

In the present study, citrated plasma often coagulated at higher centrifugation speeds, or incubation at higher temperatures (37°C). For this reason PGI_2 was added to platelet suspension before each centrifugation step to inhibit platelet activation.

2.4.2 Blood Collection

When functional platelet responses are being tested, best results are obtained with fresh blood rapidly collected from drug-free healthy donors, and used immediately (Radomski et al. 1996). When platelets are refrigerated, morphological changes occur which can inhibit their normal function, and, after freezing, the platelet loss is approximately 30% (Holme 1998). Ideally blood should be collected rapidly and evenly ensuring continuous flow and minimal turbulence to avoid platelet activation and clotting. This can be facilitated by using needles and extension tubes with large diameters and by collecting from the largest veins possible. Blood should be mixed with an anti-coagulant as soon as possible after removal from the body as blood can clot within minutes. While citrated buffers are most often used as anticoagulants for platelet experiments, EDTA and heparin are other alternatives. Commonly used methods of blood collection are listed below:

2.4.2.1 VACUTAINER SYSTEMS

This method is quick and simple, and allows blood to immediately come into contact with an anti-coagulant buffer in silconized glass tubes. However, flow is turbulent and the volume of these tubes (5 – 10 mL) may be too small for some applications.

2.4.2.2 CATHETERIZATION

This method involves the insertion of a small plastic tube, which is attached to an extension set, into a vein. Blood can be drawn from the extension set using syringes or vacutainer systems. This method is advantageous for multiple sampling over a short time period as it reduces the number of needle insertions for the subjects. However, the narrow diameter of the tube results in slow blood flow, and the stagnant flow of blood between draws results in the formation of small clots in the tubing. Between multiple draws, the extension tubing and catheter are rinsed with saline or heparin to remove blood clots that may block blood flow. Nonetheless, these rinses do not always clear away tiny clots, which may not block the flow of blood but will cause platelets passing through the tubes to stick to these sites. This will result in either a substantially decreased platelet recovery upon isolation and/or the formation of platelet clots (activation) during the experiment. In general, this method of blood collection should be avoided for platelet studies.

2.4.2.3 BUTTERFLY SETS AND SYRINGES

The use of a butterfly needle connected to an extension tube, both available in several sizes, allows a smooth and even blood flow into a large range of syringe sizes (1cc to 60cc). Anti-coagulant can be added to the syringe if a large volume is being drawn, or blood can be immediately transferred into polypropylene tubes containing anti-coagulant. If a larger volume is needed, stop-cocks can be attached to the butterfly tubing to allow the attachment of multiple syringes. However, it is important to keep a constant flow throughout the draw, and thus detachment of syringes from the collection sets should be done as soon as possible. This was the method of blood collection chosen for this study due to the large volume of blood being collected and because platelet activation (clot formation) was frequently encountered using the alternate collection methods.

2.4.3 Platelet Isolation

Effective separation of PRP from red blood cells can be obtained by centrifugation at low speeds, ranging from 140g to 400g for 5 to 30 minutes at room temperature. (In this study whole blood was centrifuged at 375g for 20 minutes at room temperature). Citrated buffers, which do not adequately inhibit platelet activation brought on by this force, require the addition of another anti-coagulant such as PGI_2 (0.06 ug/mL), a potent anticoagulant with a short half-life (Radomski et al 1996). Platelets can be pelleted out of the PRP by centrifugation at higher speeds, ranging from 600g to 2300g for 5 to 15 minutes. (In this study PRP was centrifuged at 2240g for 10 minutes at room temperature). Again, the addition of a stronger anti-coagulant is needed prior to centrifugation to prevent platelet activation. PGI_2 (0.3 μg/mL) works well to inhibit activation at this step (Radomski et al 1996). A small pellet of platelets is present at the bottom of the tube and the PPP is gently removed. The platelet pellet is then re-suspended in buffer by gently withdrawing and ejecting buffer against the side of the tube near the pellet, but not directly at the pellet. With this procedure it may take several minutes to obtain an even cell suspension.

2.5 Dye Incubation

The citrated platelet suspensions were counted using a Coulter Counter, adjusted to a concentration of 3.5×10^8 cells per mL with the citrated HEPES buffer, then incubated in the dark with 3 μM Fura-2AM and 30 μL PF-127 (10%) per 1 μmol Fura-2AM stock solution at 37^0C for 30 minutes. Following incubation, 0.3 μg/mL PGI_2 was added to the suspension and centrifuged at 2240g for 10 minutes at room temperature.

The supernatant was discarded, and the pellet was washed three times by gently layering and removing 1 mL HEPES buffer on top of the pellet. Platelets were then re-suspended in 5-7 mL of HEPES buffer, counted and adjusted to a concentration of 1.0×10^8 cells per mL. All platelet suspensions were left to sit for 10 minutes at room temperature in Ca^{2+}-containing HEPES before adding drugs or beginning measurements.

2.5.1 Dye Loading Conditions

Many protocols are described in the literature to load platelets with Fura-2. In determining the protocol to use in this study, several factors that affect dye loading efficiency and the quality of measurements obtained, were considered and are discussed in the following sections.

2.5.1.1 INCUBATION TIME AND DYE CONCENTRATION

Incubation times of 15 to 60 minutes are usually used for dye loading as these are long enough to produce an adequate signal to noise ratio, and short enough to minimize dye leakage out of cells. A range of dye incubation times from 15 to 30 minutes (3 μM Fura-2AM, 37°C with platelet suspensions) shows that an increase in incubation time correlates with an increase in fluorescence intensity, indicating increased dye loading occurred (Figure 7). Conversely, a lower fluorescence intensity is obtained with an incubation time of 45 minutes compared to 30 minutes (3 μM Fura-2AM, 37°C), indicating that longer incubation times may lead to dye leakage and decreased loading (Figure 8). However, the dye concentration and incubation temperature can affect the incubation time needed and should be considered in parallel.

The use of the dispersing agent (non-ionic, non-denaturing) detergent Pluronic-F-127 (PF-127) was found to increase dye loading as measured by an increase in fluorescence intensity in this study when added to cell suspensions during Fura-2 incubation (data not shown, similar trend to Figure 7). PF-127 helps solubilize Fura-2AM in aqueous solutions and facilitates it in crossing the cellular membrane.

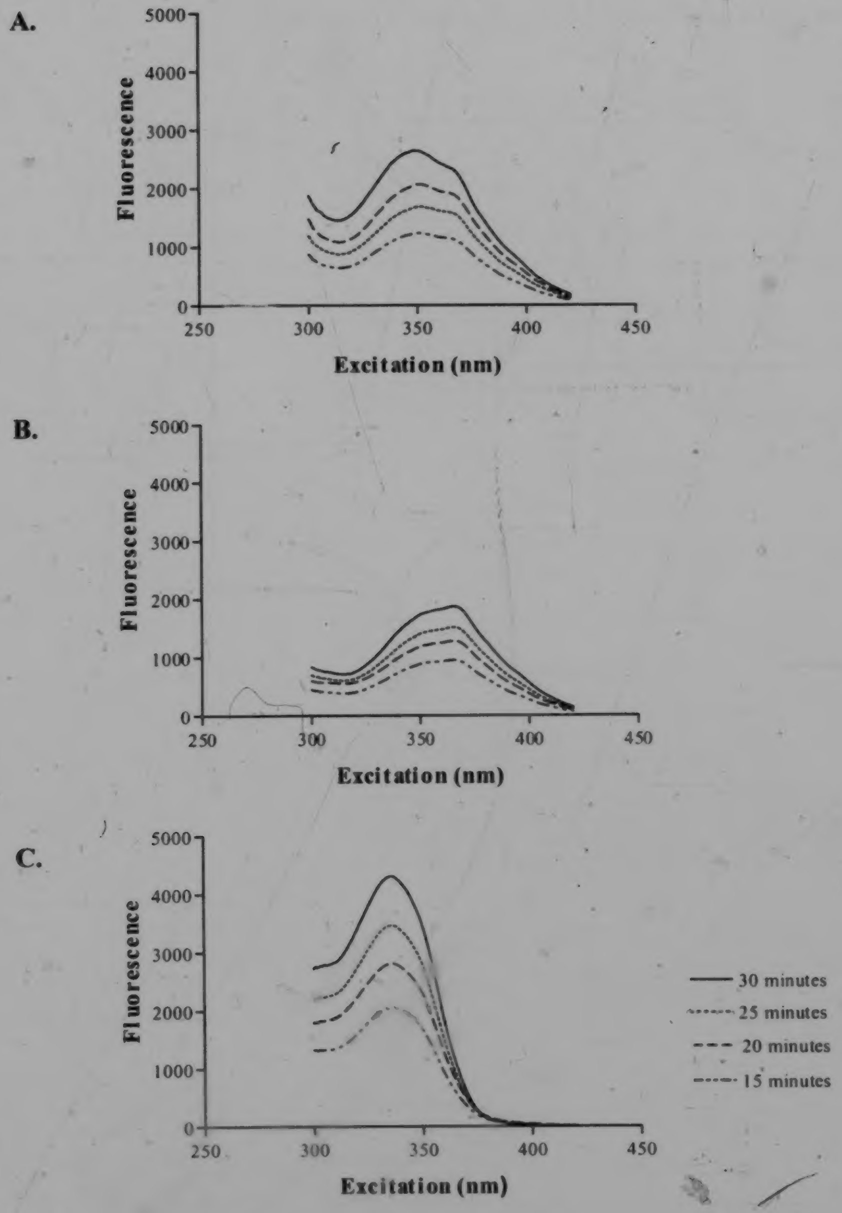

Figure 7 - Effects of Incubation Time on Fura-2 Loading in Platelets

Platelets were [illegible] incubated at 37°C for 30 minutes. Fura-2AM (3μM) was added after 0, 5, 10 and 15 minutes. A: Baseline, B: F_{min} after the addition of 20 μL SDS (10%) and 6.25 mM EGTA, C: F_{max} after the addition of 7.5 mM $CaCl_2$. All panels show an increase in fluorescence intensity with an increase in incubation time, suggesting an increase in dye loading.

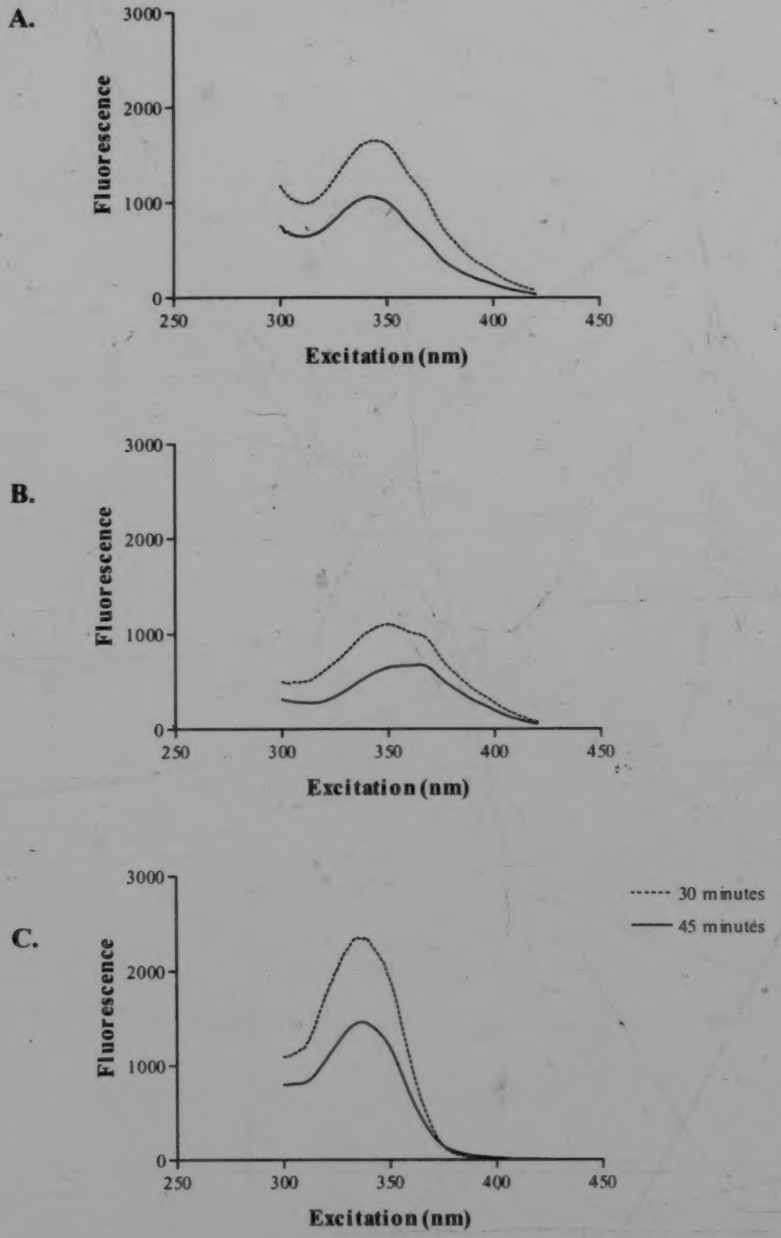

Figure 8 - Effects of Incubation Time on Fura-2 Loading in Platelets

Platelets were all incubated at 37°C for 45 minutes. Fura-2AM (3µM) was added after 0 and 15 minutes. A: Baseline, B: F_{min} after the addition of 20 µL SDS (10%) and 6.25 mM EGTA, C: F_{max} after the addition of 7.5 mM $CaCl_2$. All panels show an increase in fluorescence intensity with a decrease in incubation time, suggesting that dye loading has decreased from 30 to 45 minutes.

The acetoxymethyl ester form of Fura-2 (Fura-2AM) is usually incubated with platelets at concentrations ranging from 0.5 to 5 μM, with 2 to 4 μM being reported most often in the literature. It is important to keep the concentration of Fura-2 as low as possible, as excess intracellular dye can be detrimental to the cell, causing Ca^{2+} buffering, cellular toxicity and intracellular compartmentalization (Takahashi et al. 1999). Higher concentrations of Fura-2AM used to load platelets has also been associated with a decrease in thrombin-induced platelet Ca^{2+} responses, compared to loading at lower doses (Fowler and Tiger 1997). Conversely, it is important to load enough dye so that adequately high signal-to-noise and intracellular-to-extracellular dye ratios are obtained. Generally, increasing the concentration of the dye in the incubation media increases the dye loading and fluorescence intensity obtained.

Increasing both incubation time and dye concentration is associated with an increase in dye loading and fluorescence intensity, as displayed in Figure 9 where the loading of concentrations of 2, 3, 4, 5 μM Fura-2AM from zero to 85 minutes was monitored in 4 minute intervals. In comparing these graphs, maximal loading appears to occur with 2 and 3 μM Fura-2AM after about 30 minutes. A plot of the 340/380 ratio peaks versus time (Figure 10) illustrates that over time, Fura-2AM is loaded into cells, the ester groups is cleaved off, and free Fura-2 is released and binds to Ca^{2+}, hence the reason for the increase in the 340 signal. There is a lower 340/380 ratio overall with higher dye concentrations, because there is excess Fura-2 which stays in the unbound form, and so a greater signal is obtained at 380 nm decreasing the ratio.

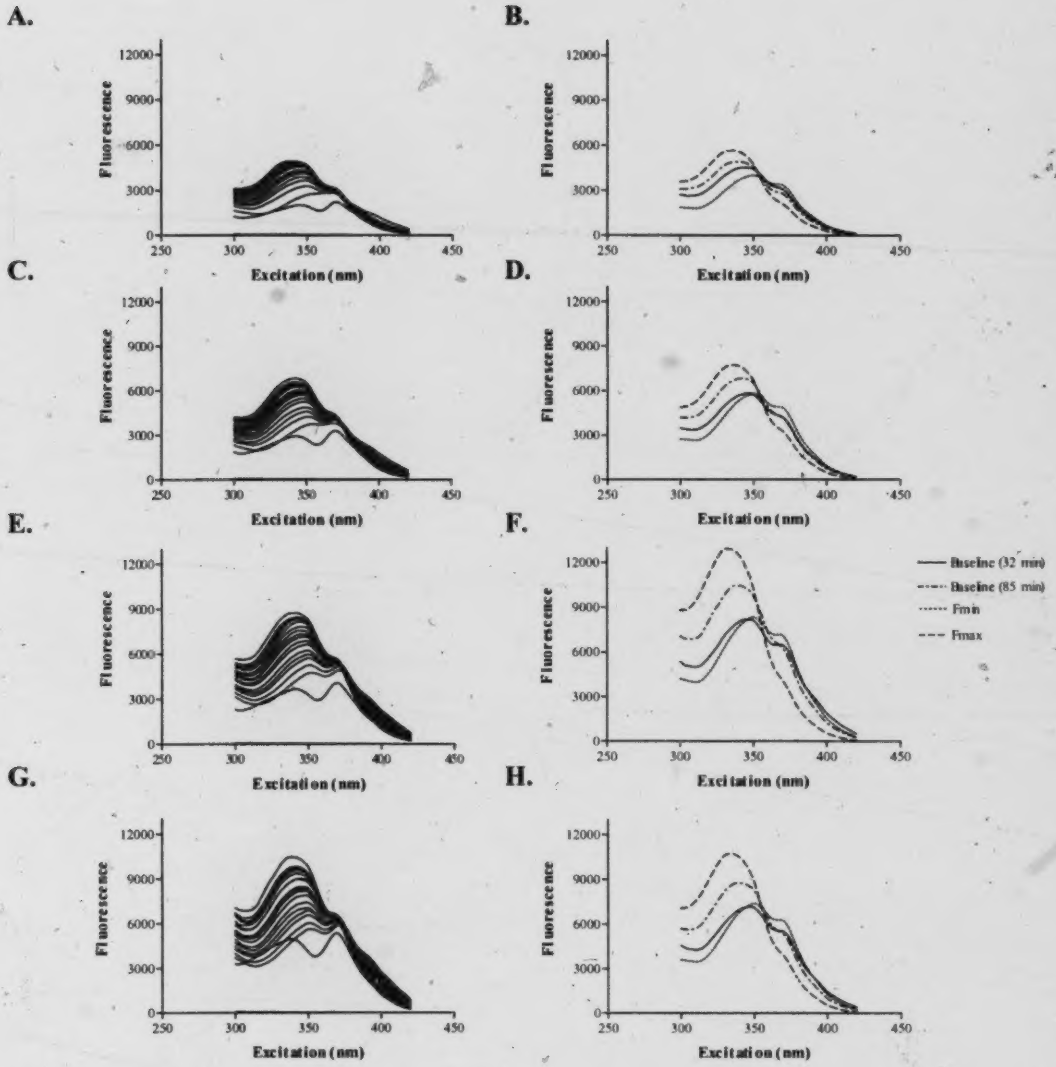

Figure 9 - Effects of Dye Concentration and Time on Fura-2 Loading in Platelets

Platelets were loaded with Fura-2AM at 37°C at concentrations of 2μM (A, B), 3 μM (C,D), 4 μM (E, F) and 5 μM (G, H). The bottom scan in each graph corresponds to time zero, while there is 4 minutes elapsed between each subsequent scan. Panels B, D, F, H show excitation spectra at 32 minutes and 85 minutes after dye loading, and calibrated with 20 μL SDS (10%) and 6.25 mM EGTA (F_{min}) followed by 7.5 mM $CaCl_2$ (F_{max}). All spectra indicate that dye-loading was increased with time and increasing concentrations. Panels A and C indicate that saturation of signal may occur, suggesting that the cells are loaded to their maximum at concentrations of 2 or 3 μM.

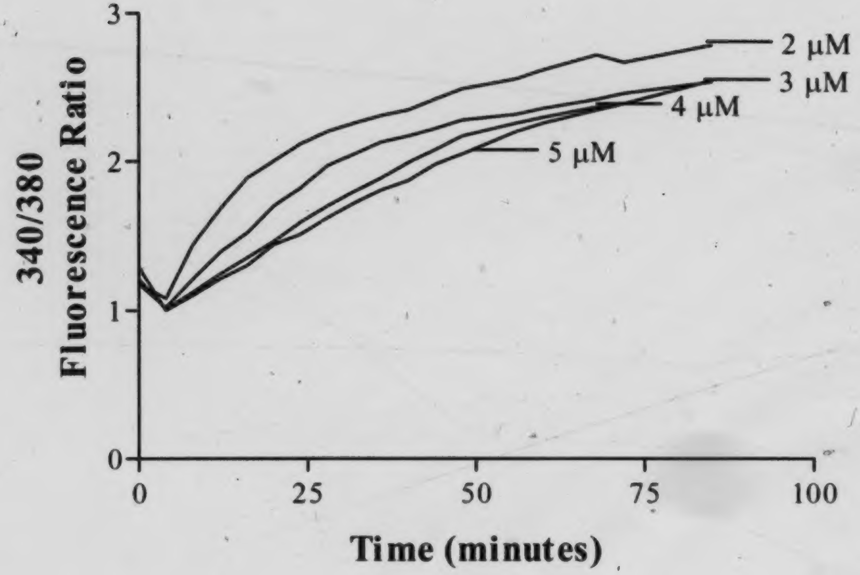

Figure 10 - 340/380 Fluorescence Ratio of Platelets Loaded with 2, 3, 4 or 5 μM Fura-2AM from zero to 85 minutes at 37°C.

This is a graph of the 340 and 380 peaks from the excitation spectra taken in Figure 9 (A, C, E, G). Over time, there is a gradual increase in the bound form of Fura-2, indicating that the dye is being loaded and the ester group is being cleaved off, allowing free Fura-2 to bind to Ca^{2+}. Increasing the concentration of Fura-2AM leads to a greater increase in the unbound form than the bound form of the dye, hence a lower ratio.

2.5.1.2 TEMPERATURE EFFECTS ON FURA-2AM HYDROLYSIS, LEAKAGE AND INTRACELLULAR COMPARTMENTALIZATION

The loading efficiency of acetoxymethyl ester dyes is often limited by the cellular esterase activity: as the esterases become saturated, excess dye that cannot have its ester group cleaved off can easily leak out of the cell, or cross membranes into intracellular organelles. Esterase activity is optimal at physiological temperature (37°C) and thus this is usually the temperature chosen for incubations. Fura-2 (3 μM Fura-2AM) loading into platelets for up 1 hour at 25°C did not increase, suggesting that the esterase activity was low (Figure 11). Further, after one hour, Fura-2 was insensitive to $MnCl_2$ or $CaCl_2$ suggesting that either all the Fura-2 was saturated or there was lingering partially hydrolyzed Fura-2, which was insensitive to Ca^{2+}, and indicating that dye loading was inefficient at room temperature. The decrease in overall fluorescence over time is likely due to photobleaching from repeated scanning (Roe et al. 1990).

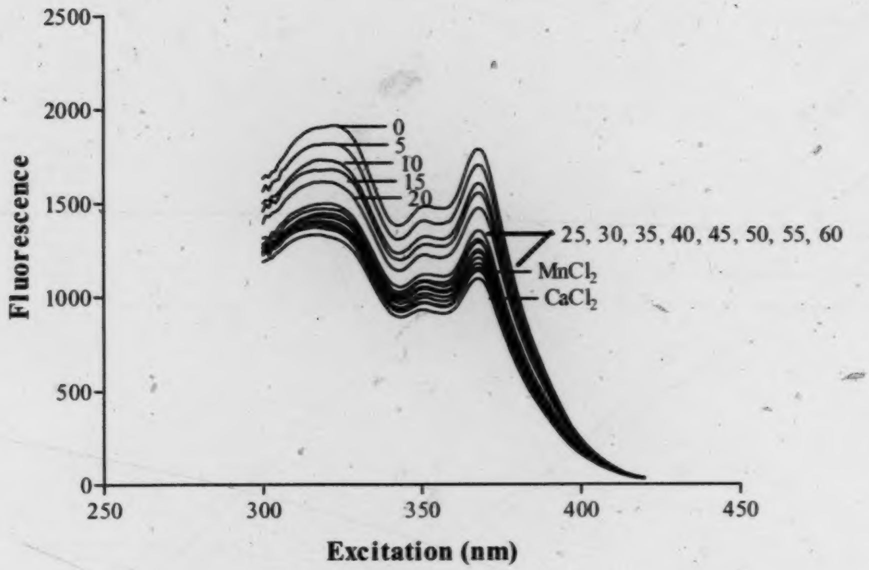

Figure 11 - Spectra of Platelets Loaded with 3 μM Fura-2AM at 25°C

Platelet suspensions scanned immediately after addition of Fura-2AM and every 5 minutes thereafter. Unlike platelets loaded at 37°C (Figure 9) there is little change in the 340/380 ratio at 25°C. Further, after one hour, Fura-2 was insensitive to 80 μM $MnCl_2$ or 6.25 mM $CaCl_2$ suggesting that any free Fura-2 present was saturated with Ca^{2+}. This suggests that Fura-2 loading is less efficient at 25°C. The decrease in florescence over time is likely due to photobleaching from repeated scanning.

Conversely, if temperatures are too high, dye leakage may increase as some cells actively transport the charged form of Fura-2 out of the cell via anionic transporters located in the plasma membrane (Virgilio et al. 1988). It is thus important to minimize the time and temperature of the experiments after the dye has been loaded into cells. Preliminary experiments in this study show that while dye leakage was apparent, it did not appear to interfere with or alter agonist-induced measurements of platelet Ca^{2+} within the 90 minutes that samples were measured after dye loading, nor did it significantly increase across time. Dye leakage was assessed after 1 hour of dye loading using manganese chloride ($MnCl_2$) which replaces Ca^{2+} bound to extracellular Fura-2 due to the greater affinity of Fura-2 for Mn^{2+} over Ca^{2+}, quenching fluorescence at the bound peak (340 nm), and EGTA, which chelates extracellular Ca^{2+}, hence releasing Fura-2 (Figure 12). $MnCl_2$ quenches extracellular Fura-2 that has leaked out of the cell and is contributing the bound fluorescence at the 340 nm wavelength. EGTA then chelates extracellular Ca^{2+} and manganese ions (Mn^{2+}), releasing free Fura-2 and resulting in an increase in the unbound (380 nm) signal. Lysing the cells with SDS causes chelation of all Ca^{2+} in the sample by EGTA and results in a release of Ca^{2+} from all Fura-2. The lack of increase in the unbound form of Fura-2 found at this point suggests that Mn^{2+} may interfere by binding to free Fura-2 and quenching the 380 signal. Thus, $MnCl_2$ was only used for protocol development and not in determination of Ca^{2+} concentrations because of this potential interference with signal calibrations (Figure 12).

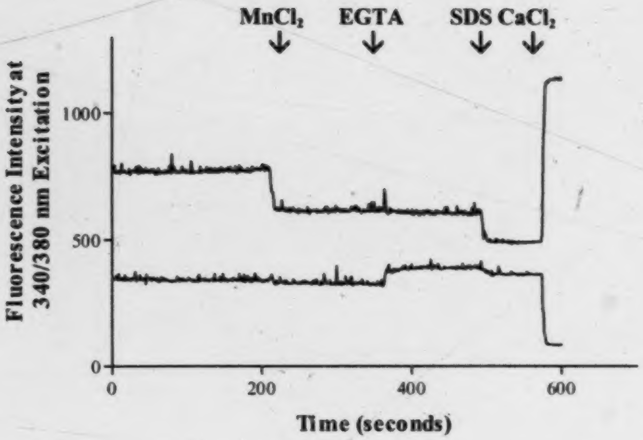

Figure 12 - Assessment of Dye Leakage in Platelets Using $MnCl_2$ and EGTA after 1 hour of Loading with Fura-2AM

Platelets loaded with 3 μM Fura-2AM at 37°C for 30 minutes. After 1 hour the 340 nm signal is decreased by the addition of 80 μM $MnCl_2$. This is presumably due to replacement of Ca^{2+} by Mn^{2+} on extracellular Fura-2 that has leaked out of the cell, resulting in a quenching of the signal. No quenching of the 380 signal is seen at this point, indicating that all indicator outside of the cells is in the bound form. After the addition of excess EGTA (6.25 mM), all Ca^{2+} and Mn^{2+} outside the cell will be chelated (EGTA binds Ca^{2+} with a greater specificity), releasing free Fura-2, hence an increase in the unbound (380 nm) signal occurs. Upon the addition of SDS (20 μL, 10%), which lyses the cells, EGTA is exposed to all Ca^{2+} in the sample and results in a release of Ca^{2+} from all Fura-2. The lack of increase in the unbound form of Fura-2 at this point suggests that Mn^{2+} may interfere by binding to free Fura-2 and quenching the 380 signal. High concentrations of $CaCl_2$ (6.25 mM) are then added to saturate the indicator and obtain a large increase in bound Fura-2 and a large decrease in unbound Fura-2.

There are two concerns if dye loading is incomplete or inefficient: (i) incomplete hydrolysis of the ester group and (ii) intracellular compartmentalization. If there is incomplete hydrolysis of the ester group, the unhydrolyzed ester can interfere with measurements, either by on-going hydrolysis of the ester group throughout experiments, or by interference with the fluorescent signal (Oakes et al. 1988). The fluorescence spectrum of Fura-2AM (1 μM) measured in low Ca^{2+} HEPEs buffer occurred at excitation wavelengths below those of both bound and unbound free-Fura-2 (< 250 nm), and was found to be insensitive to calcium (Figure 13).

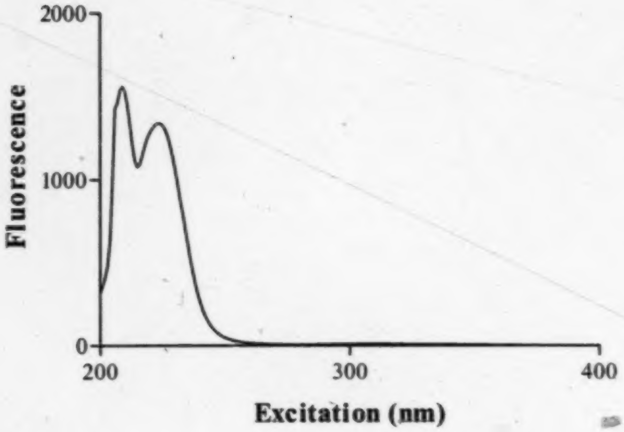

Figure 13 - Spectra of 1 μM Fura-2AM in low Ca^{2+} HEPES buffer

Spectra of Fura-2AM (1 μM) shows that the unhydrolyzed indicator will contribute little interference to the 340 and 380 peaks of the Ca^{2+}-sensitive form of Fura-2. The unhydrolyzed form of Fura-2 is insensitive to the addition of 300 mM $CaCl_2$. Low Ca^{2+} refers to Ca^{2+} impurities in HEPEs and water used to prepare the buffer, no excess $CaCl_2$ was added.

It is important to consider that if the ester group is not rapidly cleaved off of intracellular Fura-2AM, the indicator can continue to cross intracellular membranes easily in the AM form. Esterases present in organelles can then cleave off the AM group resulting in compartmentalization of the charged form of the indicator in intracellular organelles (Roe et al. 1990, Takahashi et al. 1999). Intracellular compartmentalization was assessed in preliminary experiments by selectively lysing the plasma membrane with low concentrations of digitonin (10 μM) to release cytosolic Fura-2, then lysing remaining membranes using SDS to release Fura-2 in organelles (Emuas et al. 1986, Roe et al. 1990) (Figure 14). There was a small decrease in the 340 nm signal after the addition of SDS in the presence of EGTA, suggesting that some of Fura-2 may have become trapped inside organelles, however this appears to be negligible.

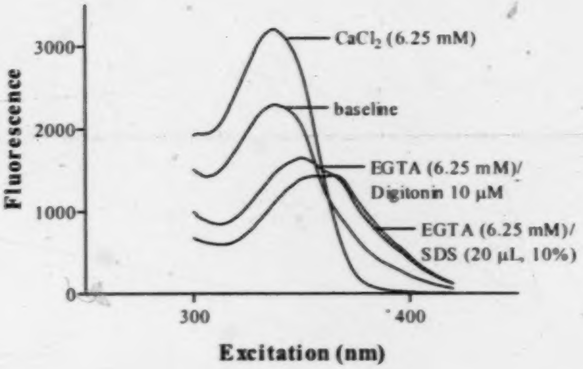

Figure 14 - Intracellular Compartmentalization of Fura-2

Platelets incubated with 3 μM Fura-2AM for 30 minutes at 37°C. Scans were performed at baseline and after the addition of EGTA (6.25 mM)/digitonin (10 μM), SDS (20 μL, 10%) (in the presence of EGTA) and then CaCl$_2$ (6.25 mM). A small decrease in the 340 nm signal after the addition of SDS suggests that a small, negligible amount of Fura-2 became trapped in intracellular organelles.

2.5.1.3 PLATELET CONCENTRATIONS

As previously mentioned, platelet concentrations that are greater than physiological concentrations (1.5 to 4.0 x 10^8 cells/mL) are more likely to become activated. Another concern is that inconsistent platelet concentrations between samples can result in variations in agonist-induced Ca^{2+} influx probably due to uneven dye loading. For example increased cellular concentration can facilitate increased dye loading, leading to cellular toxicity (Fowler and Tiger 1997) (Figure 15). A concentration of 3.5 x 10^8 cells/mL was chosen in this study for the incubation step as it allowed for adequate dye loading. A concentration of 1.0 – 2.0 x 10^8 cells/mL is usually adequate to obtain a good signal-to-noise ratio in fluorometric measurements. A concentration of 1.0 x 10^8 cells/mL produced an acceptable signal-to-noise ratio in this study and was used in order to maximize the number of samples available for measurements (at least twelve-2 mL samples were obtained from each blood draw).

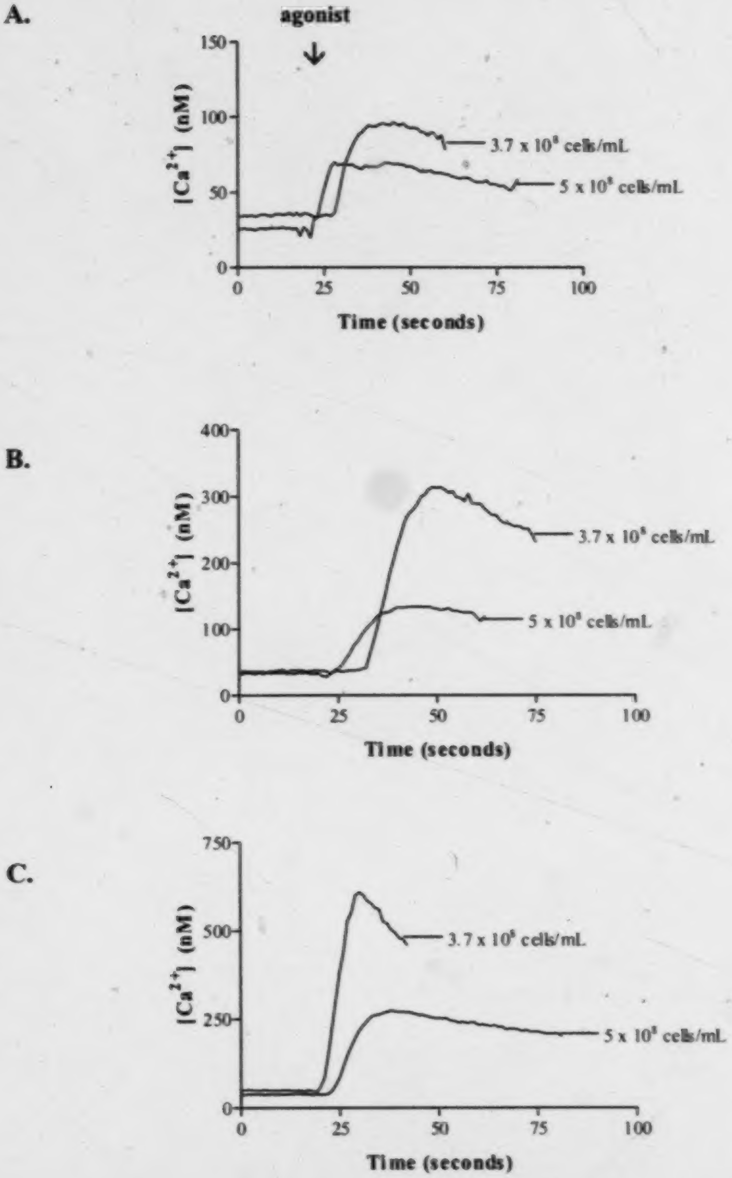

Figure 15 - Effects of Platelet Concentration on Agonist-Induced Changes in [Ca^{2+}]

Platelets from the same subject incubated with Fura-2AM (3 μM) at 37°C for 30 minutes at two different cellular concentrations (3.7 x 10^8 cells/mL and 5.0 x 10^8 cells/mL). All fluorometric measurements were performed with 1.0 x 10^8 cells/mL. Agonists added for Panel A: 10 μM 5-HT, Panel B: 0.015 U/mL thrombin and Panel C: 0.040 U/mL thrombin. A reduced agonist-induced increase in [Ca^{2+}] is found with higher platelet concentrations during dye incubation.

2.5.1.4 INTERFERENCE FROM HEAVY METALS AND pH

Heavy metals, such as $ZnCl_2$, can bind to and quench Fura-2 fluorescent signals (Roe et al. 1990). Heavy metal interference was assessed by the addition of micromolar concentrations of $ZnCl_2$, or N,N,N',N'-tetrakis(2-pyridyl-methyl)ethylenediamine (TPEN), a heavy metal chelator, and these were not found to significantly interfere with either the 340 nm or the 380 nm excitation spectra of cell-free solutions of free Fura-2 (pentapotassium salt) (Figure 16). However, there was a slight increase in the 380 nm (unbound) Fura-2 signal after the addition of up to 60 µM TPEN following the SDS/EGTA end calibration in platelets (Figure 17). This suggests that TPEN is binding to an unknown divalent cation, which may have been quenching free Fura-2. This may have been Mg^{2+}, which is present in cells at a concentration of about 1 mM. The pH value in all experiments was kept constant (7.3), as both Fura-2- and EGTA- Ca binding are quite sensitive to changes in pH (Groden et al. 1991) (Figure 18).

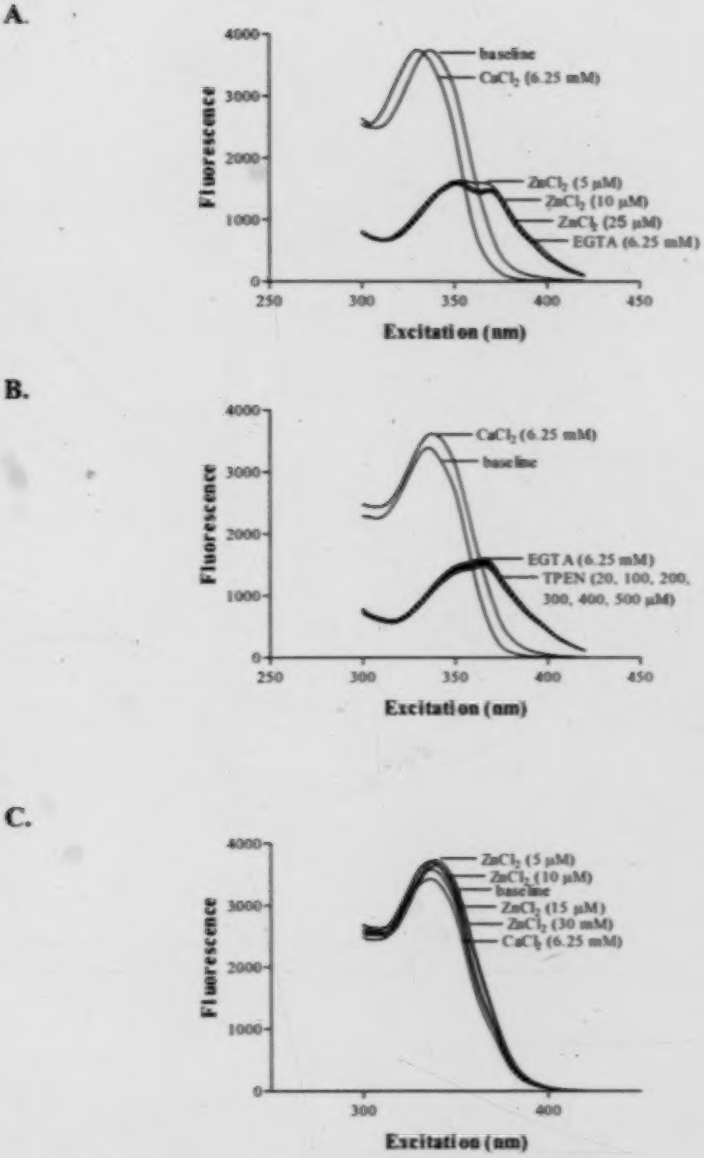

Figure 16 - Effects of Heavy Metals on free Fura-2 Excitation Spectra (Cell-Free)

Fura-2 pentapotassium salt in low Ca^{2+} HEPEs buffer (no added Ca^{2+} to buffer). Panel A: $ZnCl_2$ up to 25 µM does not alter the Fura-2 380 nm peak. Panel B: TPEN up to 500 µM does not alter the Fura-2 380 nm peak. Panel C: $ZnCl_2$ up to 30 µM does not alter the Fura-2 340 nm peak. These results suggest that $ZnCl_2$ does not interfere with the Fura-2 signal, and that negligible amounts of heavy metal impurities are found in the working buffers.

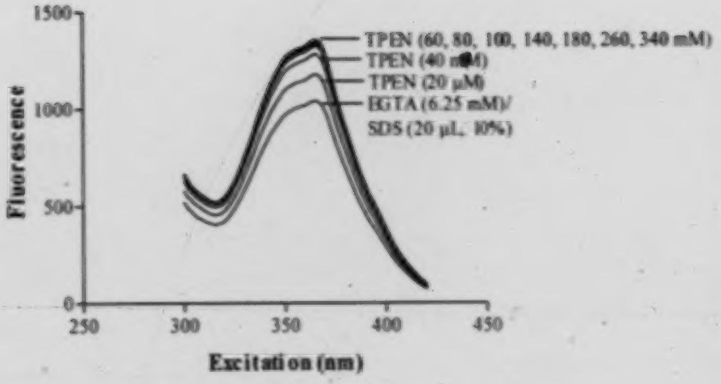

Figure 17 - Effects of Heavy Metal Chelator TPEN on Fura-2 380 nm signal During End-Calibration of R_{min} in Platelets

Platelets incubated with 3 µM Fura-2AM for 30 minutes at 37°C. After the calibration of platelets with SDS (20 µL, 10%) and EGTA (6.25 mM), various doses of TPEN were added. There appears to be something present that quenches free Fura-2, as this signal is increased upon the addition of TPEN.

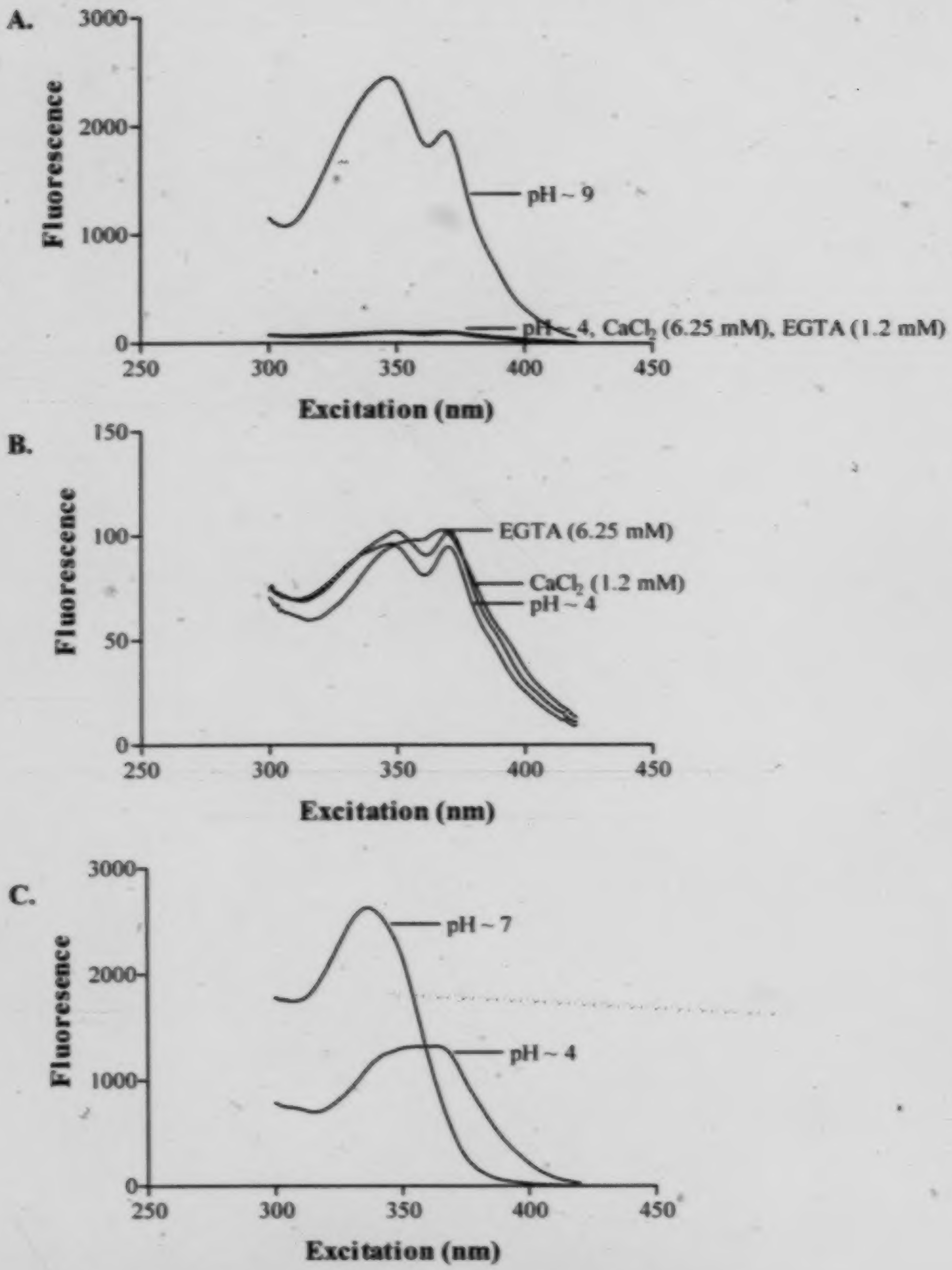

Figure 18 - Effects of pH on Fura-2-Ca and EGTA-Ca Binding

Panel A shows that the binding of EGTA is highly sensitive to pH, with an increasing affinity for Ca^{2+} at higher pH. The affinity of Fura-2 for Ca^{2+} is nearly lost low at acidic pH as the addition of $CaCl_2$ does not alter the 340 nm spectra (B) and the bound Fura-2 spectra shifts to the unbound spectra (Fura-2 releases Ca^{2+}) (C).

2.5.1.5 WASHED PLATELET SUSPENSIONS VERSUS PRP

There are advantages to using washed platelet suspensions, compared to PRP, for loading dye into platelets. In washed suspensions plasma proteins and enzymes, which can interfere with dye loading, are removed; blood plasma contains esterases which can prematurely cleave the acetoxymethyl group so the indicator can no longer cross the plasma membrane to get into the cell. In washed suspensions, the concentration of platelets, ion levels and pH can all be manipulated and kept constant between samples, while this is more difficult with PRP. For these reasons, uneven dye loading can result with the use of PRP and adds to variations in Ca^{2+} measurements. The advantage of using PRP is that there is less platelet handling and thus platelet loss and activation is minimized.

2.5.1.6 REMOVING EXTRACELLULAR DYE

Centrifugation is a relatively easy and effective method for the separation of extracellular dye from platelets. For functional studies, the platelet pellet is washed with HEPES buffer (1 mL) to remove prostacyclin and plasma proteins. This is accomplished by gently layering and removing the buffer on top of the pellet three times. The presence of PGI_2 in the following steps can obscure measurements and thus it is best to allow about 30 minutes after this step to ensure any lingering PGI_2 is degraded (Radomski et al. 1996). The platelet suspension is allowed to sit for 10 minutes in HEPES buffer to allow cellular Ca^{2+} levels to replete in case they were decreased during incubation with citrate (a weak Ca^{2+} chelator) buffer.

2.6 Drug Incubations

Platelet suspensions taken at baseline were split into 2 mL aliquots and incubated in the dark with either 1.0 μM lithium chloride, 600 μM sodium valproate, 40 μM carbamazepine, 50 ng/mL dextroamphetamine sulphate or no drug for 40 minutes at room temperature. Platelet suspensions incubated with no drug were measured for each batch of drug incubations and served as the controls. All treatment groups were measured in duplicate.

2.6.1 Drug Concentrations

Therapeutic plasma concentrations of mood stabilizers typically found in bipolar patients were used for incubations with sodium valproate and carbamazepine which range from 300 to 700 μM and 20 to 50 μM, respectively. A dose of 1.0 μM lithium chloride was used which is below therapeutic levels of 0.6 to 1.2 mM, but may be a closer representation of brain concentrations, as brain-to-plasma ratios have been reported as being less than 1. The peak plasma concentration of dextroamphetamine, ~ 50 ng/mL, reported 3 hours after administration of 0.25 mg/kg dextroamphetamine to healthy subjects reported in a previous study (Jacobs and Silverstone 1986) was chosen for the *in vitro* incubations in this study.

2.7 Fluorometry

An 8100 Series Spectrofluorometer was used for all measurements. Samples were pre-warmed to 37°C for 4 minutes in quartz cuvettes, and all measurements were made at 37°C (using a circulating water bath into the sample holder) with constant gentle stirring. All measurements were made within 90 minutes of the last re-suspension. Measurements were made in duplicate, alternating between drug-treated and untreated samples to control for any effects of time it took to carry out the scans. Fluorescence intensity was recorded against time with dual excitation at 340/380 nm and sensitivity set at 750 volts (Figures 19, 20). These are the wavelengths that correspond to Fura-2 bound to Ca^{2+} and free Fura-2, respectively. The emission wavelength was set to 510 nm as this was determined to be the wavelength at which a maximal signal was obtained. After a stable basal recording was obtained, samples were exposed to either thrombin (0.024 U/mL) (Figure 19A) or 5-HT (10 μM) (Figure 20B) using a P-200 pipette via a small injection port directly above the sample. This allowed a continuous recording of $[Ca^{2+}]$ to be obtained. The measurements were then calibrated with minimal and maximal Ca^{2+} concentrations to get a measurement of unbound and saturated Fura-2. This was done by exposing each sample to EGTA (6.25 mM) followed by SDS (20 uL, 10%) (R_{min}), followed by the addition of $CaCl_2$ (7.5 mM) (R_{max}) (again via the injection port). The ratios of the 340/380 peaks for both thrombin (Figure 19B) and 5-HT (figure 20B) were used to determine the concentrations of Ca^{2+}. A plot of time versus $[Ca^{2+}]_i$ was automatically generated by the 8100 Series Spectrofluorometer intracellular probe software program for both thrombin (Figure 21A) and 5-HT (Figure 21B) using the formula derived by Grynkiewicz et al. (1985) (Equation 2). A K_d of 224 nM was used

(Grykiewicz et al. 1985) and R_{min} and R_{max} were taken directly from the time trace readings for each sample. Basal, peak and change in $[Ca^{2+}]$ were recorded from each graph.

An excitation scan was recorded over a wavelength range of 300 to 420 nm at baseline and after R_{max} calibration for each batch of platelets (Figure 22) to ensure adequate dye loading had occurred. Excitation scans were automatically corrected for wavelength-dependent distortions in the intensity of excitation light and the efficiency with which it is transmitted, and smoothed using 15-point smoothing.

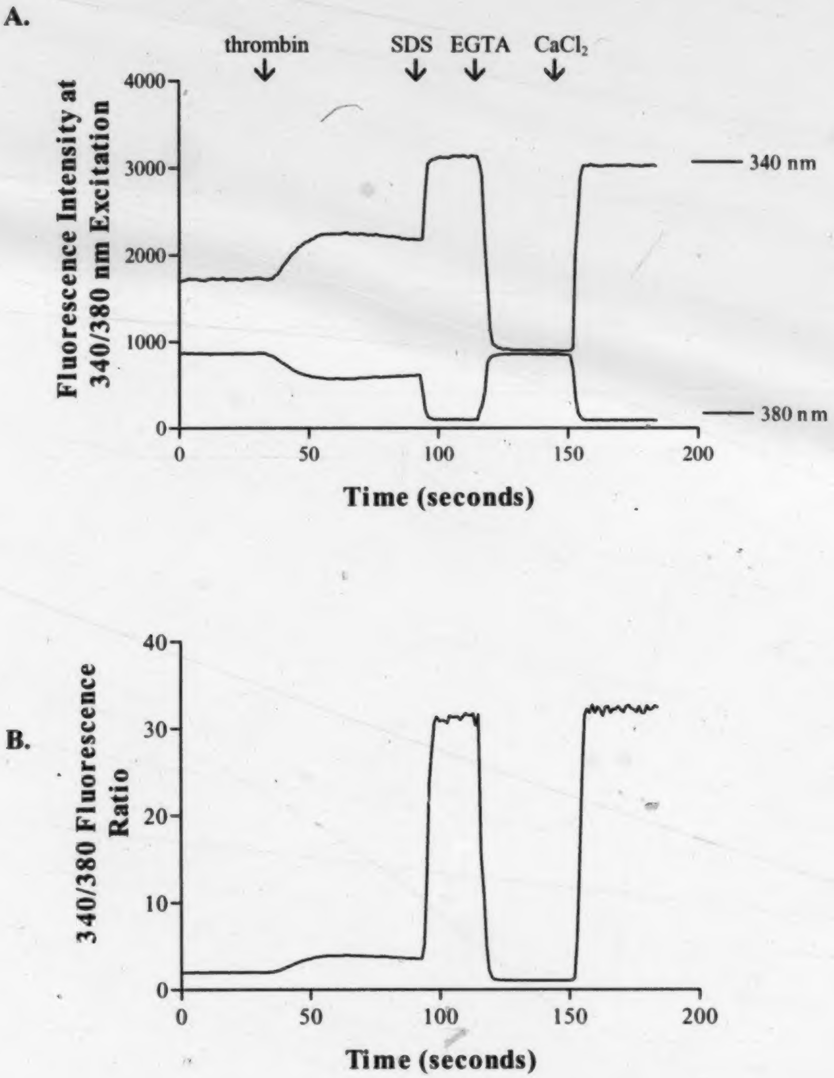

Figure 19 - Dual-Excitation Scan of Thrombin-Induced Changes in Platelet [Ca^{2+}]

Fura-2 fluorescence (37°C) at dual excitation of 340/380 time trace (A) and 340/380 fluorescence ratio (B) measured at baseline then after the addition of 0.024 U/mL thrombin, 20 µL SDS (10%), 6.25 mM EGTA, 7.5 mM $CaCl_2$.

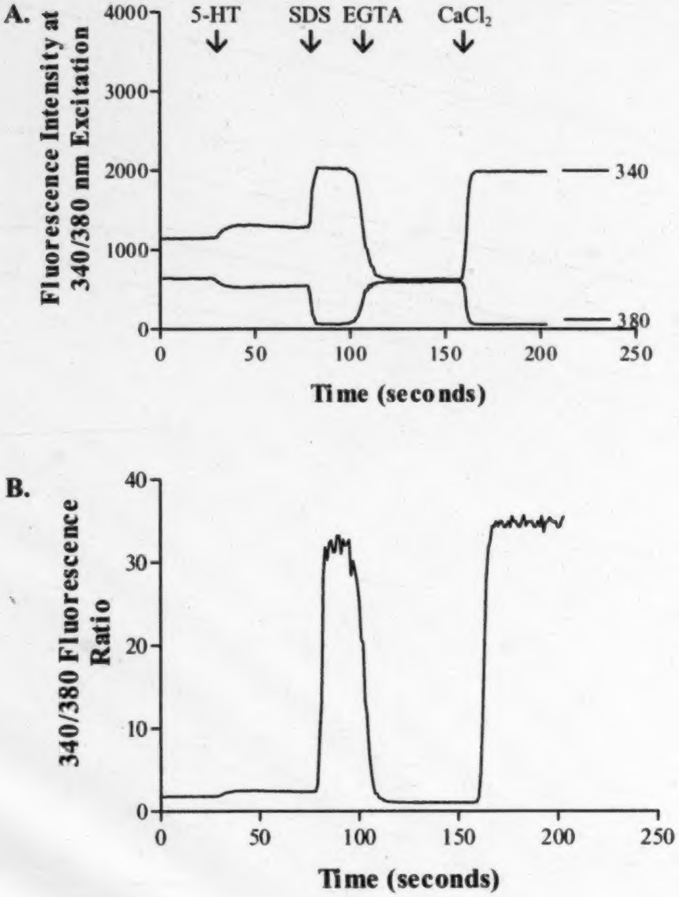

Figure 20 - Dual-Excitation Scan of 5-HT-Induced Changes in Platelet [Ca^{2+}]

Fura-2 fluorescence (37°C) at dual excitation of 340/380 time trace (A) and 340/380 fluorescence ratio (B) measured at baseline then after the addition of 10 µM 5-HT, 20 µL SDS (10%), 6.25 mM EGTA, 7.5 mM $CaCl_2$.

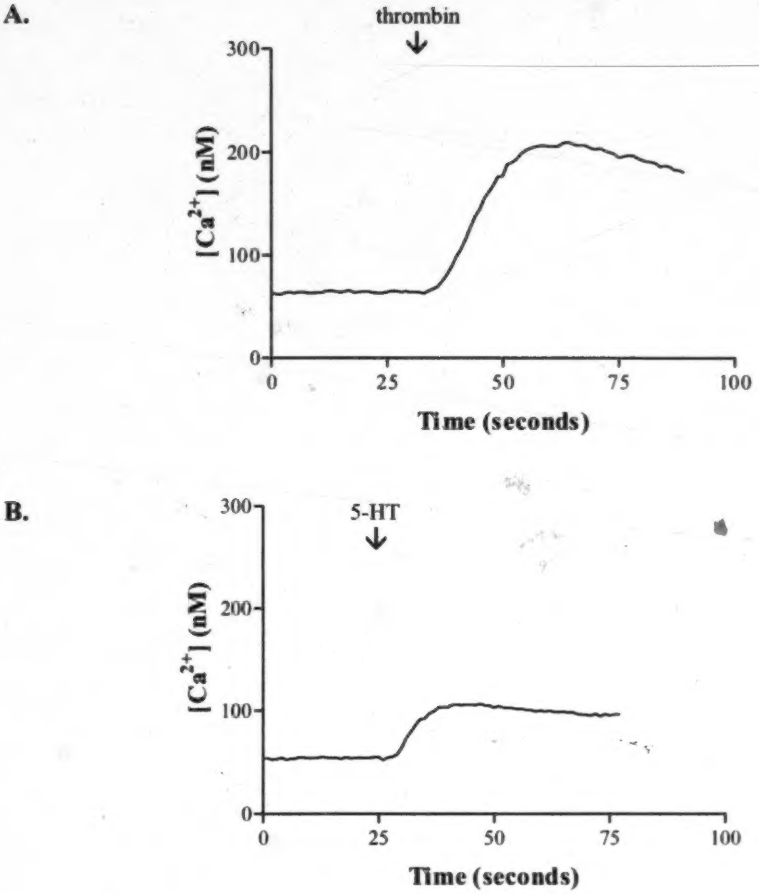

Figure 21 - Agonist-Induced Changes in Platelet $[Ca^{2+}]$

$[Ca^{2+}]$ (nM) determined in platelets at baseline and in response to .024 U/mL thrombin (A) or 10 μM 5-HT (B).

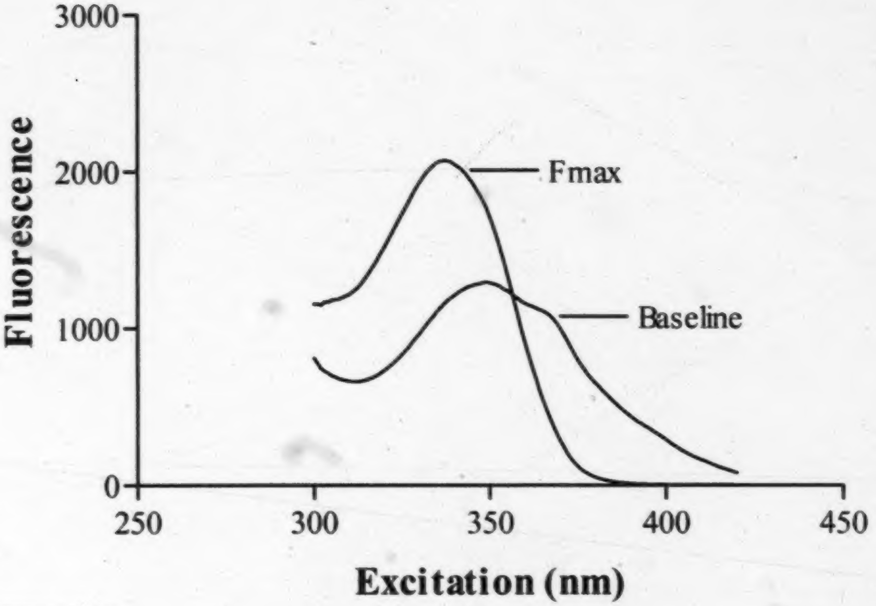

Figure 22 - Fura-2 Excitation Scans from a Platelet Suspension

Excitation scans were taken at the beginning of the experiment (baseline) and at the end of the experiment (F_{max}) for each batch of isolated platelets to ensure adequate dye loading had occurred. Excitation scans were automatically corrected from wavelength-dependent distortions in the intensity of excitation light and the efficiency with which it is transmitted, and smoothed using 15-point smoothing.

2.7.1 Determining Range of Measurements Obtainable with Fura-2 Loaded Platelets

The range of fluorescence of calcium bound Fura-2 (340 nm) was assessed by subtracting the fluorescence intensity at 340 nm at minimal Ca^{2+} concentrations (F_{min}) from that at 340 nm at maximal Ca^{2+} concentrations (F_{max}). For thrombin-induced changes in $[Ca^{2+}]$ from Figure 19A this was 3030 (F_{max}) – 900 (F_{min}) = 2130. For 5-HT-induced changes in $[Ca^{2+}]$ from Figure 20A this was 1990 (F_{max}) – 620 (F_{min}) = 1370. It was then determined where the baseline and agonist-induced fluorescence fell within this range. This information is important because a baseline fluorescence intensity that is too close to the F_{min} of Fura-2 may indicate that there is not enough dye in the cells (possibly due to excessive dye leakage), leading to incorrect measurements. Conversely, a baseline or agonist-induced fluorescence intensity that is close to the F_{max} of Fura-2 indicates that Fura-2 is nearly saturated with Ca^{2+}, and thus changes in Ca^{2+} will not be detected. Baseline (1145 at 340 nm) and 5-HT-induced (1310 at 340 nm) fluorescence values measured in Figure 21A were determined to be 38% and 50% of the fluorescence range, respectively. Baseline (1720 at 340 nm) and thrombin-induced (2255 at 340 nm) fluorescence values measured in Figure 20A were determined to be 38% and 64 % of the fluorescence range, respectively. These percentages are ideal for detecting Ca^{2+} changes.

Figures 19 and 20 show that there are variations in the fluorescence intensity measured between samples, even though the same methodology was used. These differences are probably produced by small deviations in incubation times, pipetting errors, and subject-specific platelet differences, which would result in some uneven loading. However, these differences are inconsequential as no significant variations within treatment groups were found.

2.7.2 Dye Leakage

Dye leakage was assessed in preliminary experiments (discussed in chapter 2) using $MnCl_2$ and EGTA and was found to be insignificant (Figure 12). Furthermore, since the ratiometric formula was used in determining $[Ca^{2+}]$, any variations in results due to dye leakage will have been cancelled out.

2.7.3 Autofluorescence

Fluorescence intensity of the reagents used in this assay was measured over an excitation range excitation range of 300 to 420 nm, to determine whether autofluorescence would interfere with the Fura-2 excitation spectra. Peak fluorescence intensities were all insignificant compared to the intensity of the Fura-2 intensities: HEPES buffer alone <6 and with the addition of 400 μL citrate buffer <6, 20 μL PF-127 <17, 30 μL $MnCl_2$ (8 mM) < 19, 50 μL SDS (10 %) < 31, 50 μL EGTA (0.5 M) < 17, $CaCl_2$ (0.5 M) < 20, 30 μL digitonin < 14, 30 μL TPEN < 17, 40 μL 5-HT and 50 μL methanol < 34. Fura-2AM was found not to interfere with the Fura-2 excitation scanning range of 300-420 nm (Figure 13). Furthermore, most variations in data due to autofluroesence will have been cancelled out due to the use of ratios in the formula for determination of $[Ca^{2+}]$.

2.7.4 Dose-Response Effects of Thrombin and 5-HT

Dose-response effects for a range of thrombin (0.005 U/mL to 1.0 U/mL) and 5-HT (1 to 10 μM) concentrations were determined in seven different platelet suspensions. A concentration of 0.024 U/ml for thrombin was selected because it produced signals which averaged 100% of the K_d for Fura-2, and the most accurate measurements are generally obtained in measurements which fall between 50 and 100% of the K_d (Meyer et al. 1990, Takahashi et al. 1999). The concentration of 10 μM for 5-HT has been used in similar studies, and produces a maximal signal which falls between 50-75 % of the K_d (5-HT is a much weaker agonist than thrombin of platelet Ca^{2+} influx).

2.7.5 Fura-2 K_d Determination

An experimental K_d was determined using a range of Ca^{2+} solutions prepared from stock solutions of Ca-EGTA buffers (zero and 1.2 mM $CaCl_2$), containing 1 mM free Mg^{2+} (Molecular Probes, C-3721). A 1.2 mM solution of Fura-2 pentapotassium salt (Molecular Probes, F-1200) was made using distilled water filtered through a Ca^{2+} sponge to remove any Ca^{2+} impurities (Figure 23). Portions (1.67 uL) of the Fura-2 salt solutions were added to a cuvette containing 2 mL zero Ca^{2+} solution and to another cuvette containing 2 mL of high Ca^{2+} solution (1.2 mM). Five uL Fura-2 salt were added into 6 mL of high Ca^{2+} solution. These solutions were used to prepare eleven solutions with Ca^{2+} concentrations ranging from 0 to 39.8 μM. These solutions were prepared following the "Protocol for Recipricol Dilutions" (Molecular Probes Product Sheet C-3721). Excitation scans were taken over a wavelength range of 250 to 450 nm, with emission set at 510 nm, and sensitivity set at 750, for each sample.

Fura-2 excitation scans recorded over a range of Ca^{2+} concentrations are displayed in Figure 24. These scans were recorded on two different days. The concentration of Ca^{2+} was calculated using an equation derived from the definition of the association constant of EGTA for calcium ($K_{a\,CaEGTA}$):

Equation 3:

$$\mathbf{K_{a\ CaEGTA} = [Ca\text{-}EGTA]/[Ca^{2+}_{free}][EGTA_{free}]}$$

Rearranging equation 3 to solve for $[Ca^{2+}]$:

Equation 4:

$$\mathbf{[Ca^{2+}_{free}] = K_{d\ CaEGTA}\ ([Ca\text{-}EGTA]/[EGTA_{free}])}$$

Since the $[Ca^{2+}_{free}]$ is so much smaller than that of EGTA, the concentrations of Ca-EGTA and $EGTA_{free}$ can be replaced by the ratio of the zero Ca^{2+} solution to the high Ca^{2+} solution, which includes the ratios of 0, 1:9, 2:8, 3:7, 4:6, 1, 6:4, 7:3, 8:2, 9:1 and 10.

The $K_{d\ CaEGTA}$ changes with ionic strength and temperature and thus must be corrected for accurate determination of free Ca^{2+} concentrations (Smith and Miller 1985, Harrison and Bers 1987, Harrison and Bers 1989, Groden et al. 1991, Marks and Maxfield 1991).

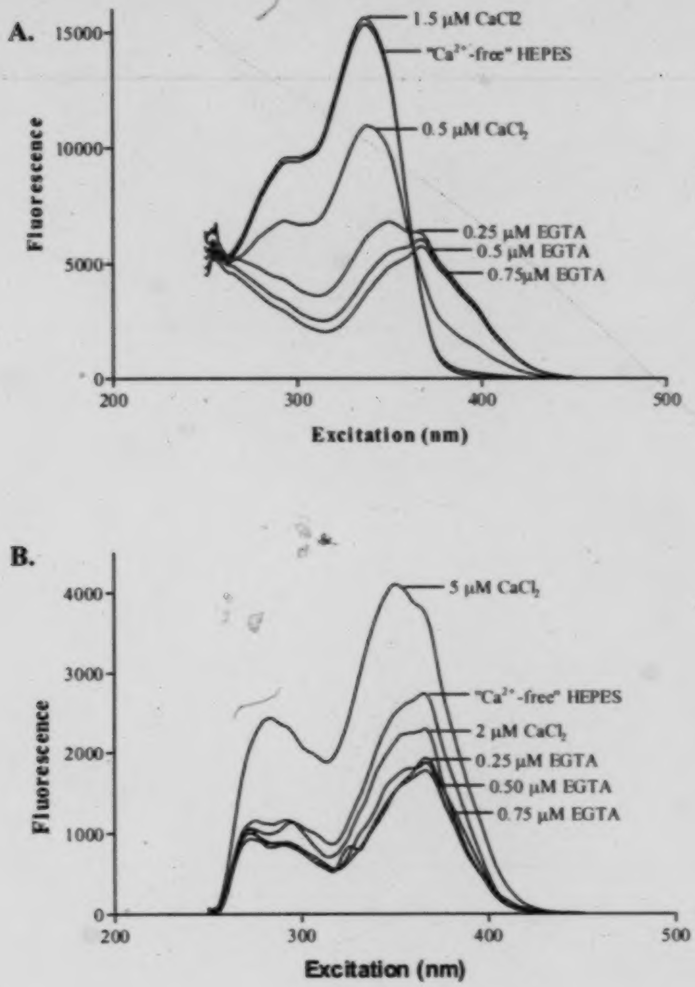

Figure 23 - Ca^{2+} Impurities in "Ca^{2+}-free" HEPES Buffer

After rinsing HEPES buffer with calcium sponge, 500 to 1000 nM of free Ca^{2+} was still present in "Ca^{2+}-free" HEPES buffer, as detected by the maximal shift to 380 nm ("unbound Fura-2") after the addition of 0.50 µM EGTA. Panel A was measured with 1.2 mM Fura-2 pentapotassium salt. Panel B was measured with 1 µM Fura-2 pentapotassium salt. This difference in concentrations is the reason for the different range of fluorescence intensities measured for each graph. Measurements made at 32°C.

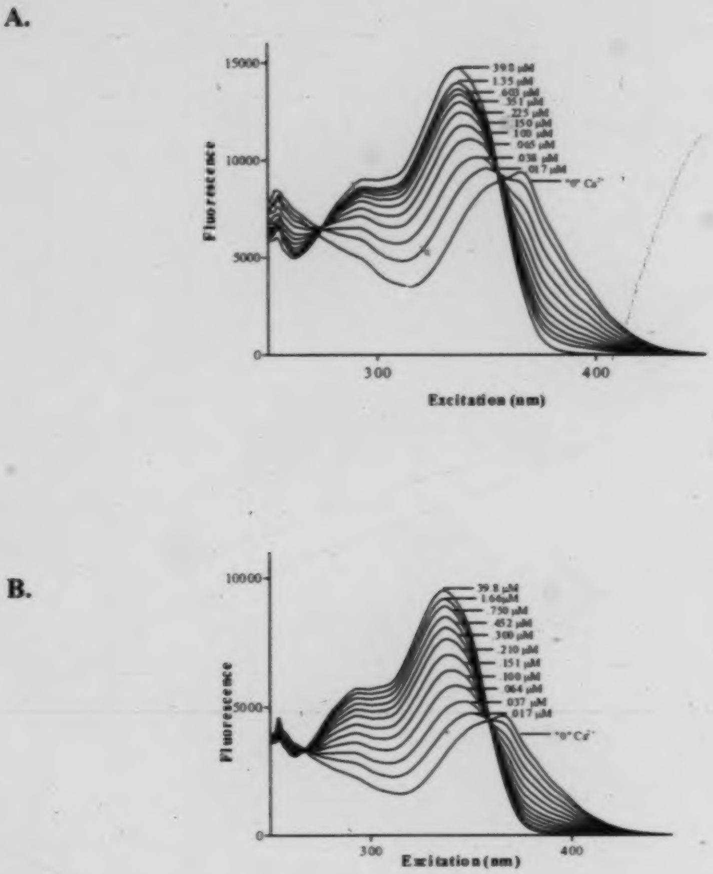

Figure 24 - Fura-2 *In Vitro* K_d Calibration

Fura-2 K_d calibrations using a range of [Ca^{2+}] from 0 to 39.8 μM at 32°C. The methods are described in text. Panel A is measured using 1.2 mM Fura-2 pentapotassium salt. Panel B is measured using 1μM Fura-2 pentapotassium salt.

Corrections for these variables were made using formulae previously described (Harrison and Bers 1987; 1989). The $K_{d\,CaEGTA}$ reported for a pH of 7.20 and I_e of 0.1 M at 20°C is 150.5 nM (Tsien and Pozzan 1989) was adjusted for an I_e of 0.001 M and a temperature of 32°C (pH 7.20). The association constant K_a is equal to:

Equation 5

$$K_a = 1/K_d$$

Thus, the K_a from the above K_d is thus equal to $6.645 \times 10^6\ M^{-1}$. The K_a was adjusted for I_e using a semi-empirical form of the Debye-Huckel limiting law (Equation 6), and then for temperature using the Van't Hoff isochore (Equation 10) (Harrison and Bers 1987, 1989):

Equation 6

$$\log K_a = \log K + 2xy(\log f_j - \log f_j')$$

where K_a is the constant after correction, K is the constant prior to correction, x and y are the valencies of the cation and anion binding species (in this case the cation is Ca^{2+} and the anion is $EGTA^{4-}$), f_j is the activity coefficient for ion j at the original ionic strength and temperature and f_j' is the activity coefficient for ion j at the desired conditions. The activity coefficent is defined by:

Equation 7

$$\log f_j = A\,[\,(I_e^{1/2}/(1+I_e^{1/2})) - bI_e]$$

where b is an empirical factor equal to 0.25 for these experiments (Miller and Smith 1984, Smith and Miller 1985), I_e is in (M) and is calculated by:

Equation 8

$$I_e = 0.5\ \Sigma C_i Z_i$$

where C is the concentration of the ionic species *i* and Z is the absolute charge of the ionic species i.

A in Equation 7 is equal to:

Equation 9

$$A = 1.8246 \times 10^6 / (\varepsilon T)^{3/2}$$

where ε is the dielectric constant of water (or alternate solvent) and T is the absolute temperature in kelvins (K). ε for water varies with temperature: 80.2 at 20°C, 76.6 at 30°C and 73.17 at 40°C. If there is also a difference in temperature between the conditions in which K and K_a were measured, this must be accounted for by calculating individual A values for each activity constant (f_j and f_j').

Correction for temperature is then made using the following formula (Van't Hoff isochore):

Equation 10:

$$\log_{10} K_a' = \log_{10} K_a + \triangle H\ [\ (1/T - 1/T') / (2.303R)\ ]$$

where △H is the change in enthalpy for EGTA and is equal to 16.6 kJ/moL, T and T' are the absolute temperatures (K) at the original and desired conditions, and R is the universal gas constant 8.314×10^{-3} expressed in kJ/K x moL.

2.7.5.1 CORRECTIONS FOR IONIC STRENGTH

The original K = 6.645×10^6 M with temperature = 20°C (293°K) and $I_e = 0.1$ M (pH 7.20). The desired conditions for K' are temperature = 32°C (305°K) and $I_e = 0.001$

M (pH 7.20). I_e was determined from Equation 8 using the following elements present in the solutions:

Mg^{2+}: C = 0.001 M, Z = 2

Cl^-: C = 0.002 M, Z = 1

The complexed Ca-EGTA will not theoretically contribute to the ionic strength. The $[Ca^{2+}]$ and the $[H^+]$ were both in the micromolar range and were thus negligible.

Equation 9 was used to determine A and A′. For A, $\varepsilon = 80.2$ and T = 293 K. For A′, $\varepsilon = 76.6$ and T = 305 K. Thus, A = 0.5065, A′ = 0.5109. These values, along with $I_e = 0.1$ M and 0.001 M were then used to determine the activity coefficients, f_j and f_j' respectively, using Equation 7.

From these calculations, $\log f_j = 0.1090$, $f_j' = 0.0155$.

Finally, Equation 6 was used to solve for K_a, with x = 2 (Ca^{2+}) and y = -4 ($EGTA^{4-}$). K_a was determined to be 2.109×10^5 M^{-1}.

2.7.5.2 CORRECTION FOR TEMPERATURE

Equation 10 was then used to correct for temperature, $K_a = 2.109 \times 10^5$, $\triangle H$ = 16.6 kJ/mol, $R = 8.314 \times 10^{-3}$ kJ/K x moL. The K_a was determined to be 2.576×10^5 M^{-1} Using $K_d = 1/K_a$, $K_{d\ CaEGTA}$ was determined to be 3882 nM.

2.7.5.3 DETERMINATION OF [$CA^{2+}{}_{FREE}$] IN CALIBRATION SOLUTIONS AND FURA-2 KD

Fura-2 K_d calibration experiments were carried out (n = 2). The $[Ca^{2+}{}_{free}]$ was determined for each solution using Equation 4 with the [Ca-EGTA]/[$EGTA_{free}$] ratio equal to 0, 1/9, 2/8, 3/7, 4/6, 1, 6/5, 7/3, 8/2, 9/1 and 10 for trial #1, and 0, 1/9, 2/8, 3/7, 4/6, 1, 5.83/4.17, 6.66/3.34, 7.5/2.5, 8.33/1.67, 9.17/0.83 and 10 for trial # 2. Two graphs were created: one in which the experimentally determined $K_{d\ CaEGTA}$ of 3882 nM was used in the calculation of $[Ca^{2+}{}_{free}]$ in the calibration solutions, and another in which a $K_{d\ CaEGTA}$ of 150.5 nM was used (determined at pH 7.20, $I_e = 0.1$ M, 20°C and 0.1 M, Tsien and Pozzan 1989). Two log-log plots of $[Ca^{2+}{}_{free}]$ (M) versus $[F\text{-}F_{min}/F_{max}\text{-}F]$ were created and the data were linearly regressed (Figures 25A, B).

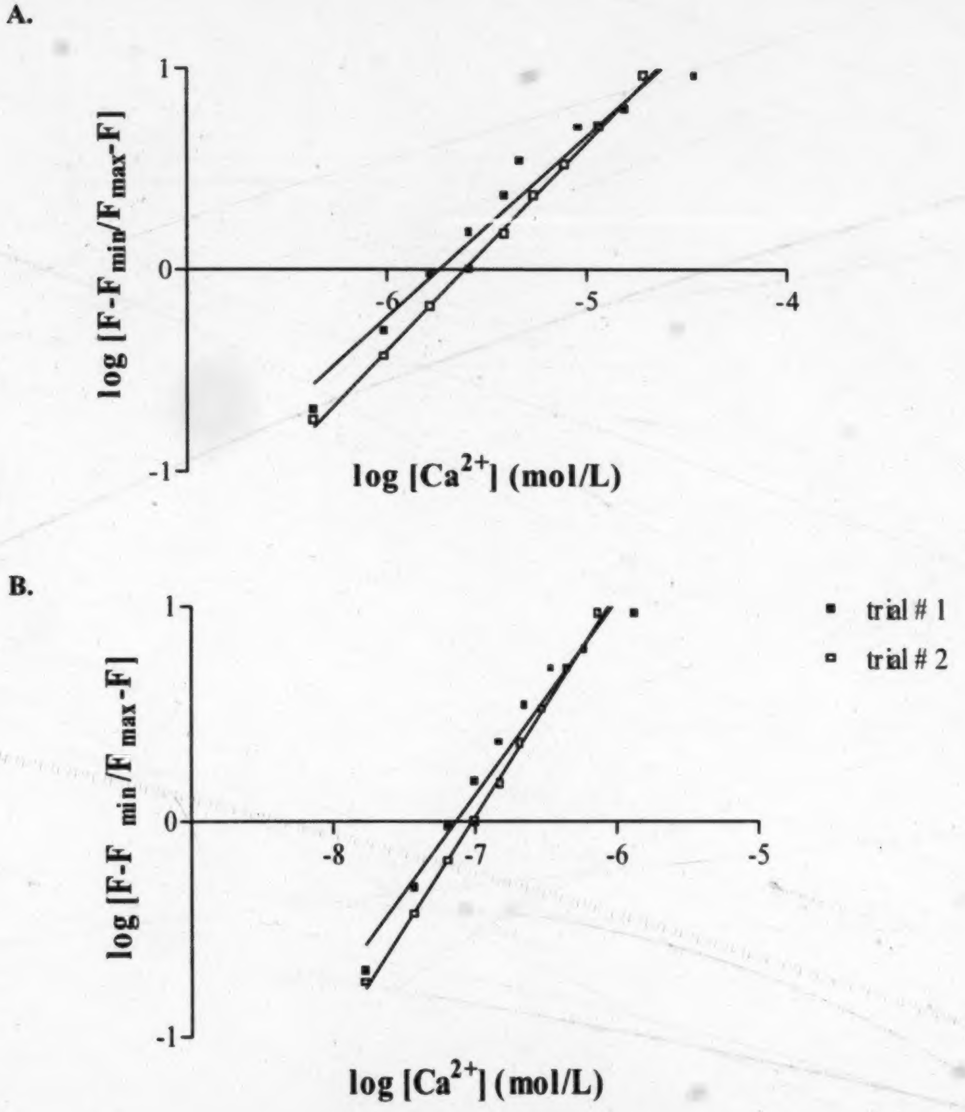

Figure 25 - Linear Regression of Fura-2 Kd Calibration

Panel A was determined using 1.2 mM Fura-2 pentapotassium salt. Panel B was determined using 1 µM Fura-2 pentapotassium salt. All measurements were made at 32°C. Trial # 1 represents $[Ca^{2+}]$ determined using the $K_{a\ CaEGTA}$ of 3882 nM determined in this study. Trial # 2 represents $[Ca^{2+}]$ determined using the $K_{a\ CaEGTA}$ of 150.5 nM previously determined (Tsein and Pozzan 1989). The x-intercepts represent the $\log_{10}K_{d\ CaFura\text{-}2}$ (M) and are –5.737 (A, trial # 1), -5.610 (A, trial # 2), -7.135 (B, trial # 1) and –7.021 (B, trial # 2). Taking the inverse log and converting to nM, the $K_{d\ CaFura\text{-}2}$ values were determined to be 1832 nM, 2455 nM, 73.28 nM and 95.28 nM, respectively.

Using the $K_{d\ CaEGTA}$ of 3882 nM, the slope of the lines for each trial were determined to be 0.899 and 1.033, which reflects the linear relationship of Fura-2-binding to Ca^{2+} (1:1). The x-intercepts were –5.737 and –5.610, which is the $\log_{10} K_{d\ CaFura\text{-}2}$ (M). Taking the inverse log and converting to nM, the $K_{d\ CaFura\text{-}2}$ was determined to be 1832 nM and 2455 nM. Using the $K_{d\ CaEGTA}$ of 150.5 nM, the slopes of the lines for each trial were determined to be 0.903 and 1.035, which reflects the linear relationship of Fura-2-binding to Ca^{2+} (1:1). The x-intercepts were -7.135 and –7.021, which is the $\log_{10} K_{d\ CaFura\text{-}2}$ (M). Taking the inverse log and converting to nM, the $K_{d\ CaFura\text{-}2}$ was determined to be 73.28 nM and 95.28 nM.

There are two major reasons for these differences. First, Ca^{2+} binding properties of Fura, similar to EGTA, are sensitive to changes in ionic composition, pH and temperature. Minor differences in experimental conditions could lead to changes in the determined K_d. The experiments in which the K_d was determined in this experiment were at pH 7.20, 1mM Mg^{2+} (ionic strength < 0.002 M), 32°C. The K_d of 224 nM reported by Grynkiewicz et al. (1985) was determined in an environment that more closely mimics that of the cytosol (115 mM KCl, 20 mM NaCl, 10 mM K-MOPS, 1 mM Mg^{2+}, pH 7.05, 37°C). This value has been repeatedly used in the literature in the determination of platelet Ca^{2+} measurements. Another concern with the K_d determination in this experiment is that the removal of Ca^{2+} from the buffer used to prepare the Fura-2 solution was not complete. Because very small concentrations of Ca^{2+} were being measured, these Ca^{2+} impurities could add to the inaccuracy of the data. For the reasons stated above, a K_d of 224 nM (Grynkiewicz et al. 1985) was used for determination of Ca^{2+} concentrations described in this study.

2.8 Statistical Analysis

All platelet suspensions that were not treated with any drug (control data) were analyzed for differences that might have occurred due to differences in the time (replicate samples) or day of measurements. Both baseline and agonist-induced values were analyzed for these changes. The results of this analysis affected the way the subsequent data was analyzed: with no significant differences in these measures, all baseline data could be analyzed between days and combined into one control group, and replicate samples could also be averaged. However, if differences were detected between time of day or day of measurement, each baseline measurement would have to be individually compared within subject. A 3-factor Repeated Measures Analysis of Variance (RM-ANOVA) was applied to all control data to test for using day (1 or 2), agonist effect (basal or peak) and sample (replicate 1 or 2) as the three factors.

Data were analyzed for effects of thrombin and 5-HT on basal Ca^{2+} concentration (basal to peak and change values), effects of drug treatment and effects of time of measurements (replicates). For this analysis a 3-factor RM-ANOVA was applied to basal and peak Ca^{2+} measurements using treatment (dextroamphetamine, placebo or control for *in vivo* drug treatments; and lithium, valproate, carbamazepine, dextroamphetamine or baseline for *in vitro* drug incubations), agonist effect (basal or peak) and sample (replicate 1 or 2) as the three factors, with the dependent variables being basal and peak Ca^{2+} concentrations. Analysis was done separately for both thrombin and 5-HT. A 2-factor RM-ANOVA was also applied to the changes in Ca^{2+} concentration (basal Ca^{2+} concentration subtracted from peak Ca^{2+} concentration) using treatment (dextroamphetamine, placebo or control for *in vivo* drug treatments; and lithium, valproate, carbamazepine, dextroamphetamine or baseline for *in vitro* drug incubations) and sample (1 or 2), with the dependent variable being change in Ca^{2+} concentration.

Greenhouse-Geisser values were used to adjust the degrees of freedom for averaged tests of significance. One-way ANOVA and Tukey post-hoc analysis were applied to resolve significant effects of treatment.

CHAPTER 3 – RESULTS

3.1 Fluorometry

Adequate dye-loading was obtained for all samples (Figure 22). Time traces were recorded for thrombin- (Figure 19A,B) and 5-HT-induced (Figure 20A,B) Ca^{2+} responses. These traces were converted to graphs of Ca^{2+} concentration for both thrombin (Figure 21A) and 5-HT (Figure 21B). (Graphs displayed in Chapter 2 – Methods, section 2.8). Samples were discarded when donors inadvertently took pain-relieving medications or platelet aggregation occurred during experiments, thus there are uneven numbers per treatment group.

3.2 Control Data

The results indicated there was a significant effect of agonist on platelet Ca^{2+} concentrations (F=13.278, df=1.00, $p < .012$), as expected. There was also a significant difference of Ca^{2+} concentrations between replicate samples (F=139.303, df=1.00, $p < .001$). Since there were no significant effects of day (F=.002, df=1.00, $p = .964$), control data from the same subjects on day 1 and day 2 were averaged. The 'n' in the following sections refers to one averaged sample per subject. There were no significant interactions between any of the groups.

3.3 Effects of *In Vivo* Dextroamphetamine on Platelet Ca^{2+} Concentrations

Thrombin induced a significant increase in platelet Ca^{2+} concentrations (F=128.040, df=1.00, $p < .001$), as expected (Figure 26). There were no significant differences between treatment groups or replicate samples on basal or thrombin-induced platelet, or change in Ca^{2+} concentrations. Basal $[Ca^{2+}]$ (nM, means ± SEM): 57.3 ± 2.4 for baseline controls; 54.8 ± 5.1 for placebo controls; and 54.1 ± 4.2 for dextroamphetamine. Peak $[Ca^{2+}]$ (nM, means ± SEM): 188.0 ± 10.1 for baseline controls; 185.0 ± 6.8 for placebo controls; and 192.3 ± 10.8 for dextroamphetamine. Change in $[Ca^{2+}]$ (nM, means ± SEM): 135.3 ± 9.3 for baseline controls; 130.3 ± 7.4 for placebo controls; and 138.2 ± 9.4 for dextroamphetamine.

5-HT-induced a significant increase in platelet Ca^{2+} concentrations (F=95.776, df=1.00, $p < .003$), as expected (Figure 27). There were no significant differences between treatment groups or replicate samples on basal or thrombin-induced platelet, or change in Ca^{2+} concentrations. Basal $[Ca^{2+}]$ (nM, means ± SEM): 66.5 ± 3.0 for baseline controls; 57.9 ± 5.1 for placebo controls; and 56.3 ± 5.2 for dextroamphetamine. Peak $[Ca^{2+}]$ (nM, means ± SEM): 114.4 ± 6.1 for baseline controls; 105.0 ± 5.1 for placebo controls; and 108.7 ± 8.0 for dextroamphetamine. Change in $[Ca^{2+}]$ (nM, means ± SEM): 47.9 ± 4.7 for baseline controls; 47.1 ± 4.6 for placebo controls; and 52.4 ± 6.1 for dextroamphetamine.

Since there were no significant differences between replicate samples in both of these analyses, the replicate samples were averaged for each treatment group graphed (Figures 26 and 27).

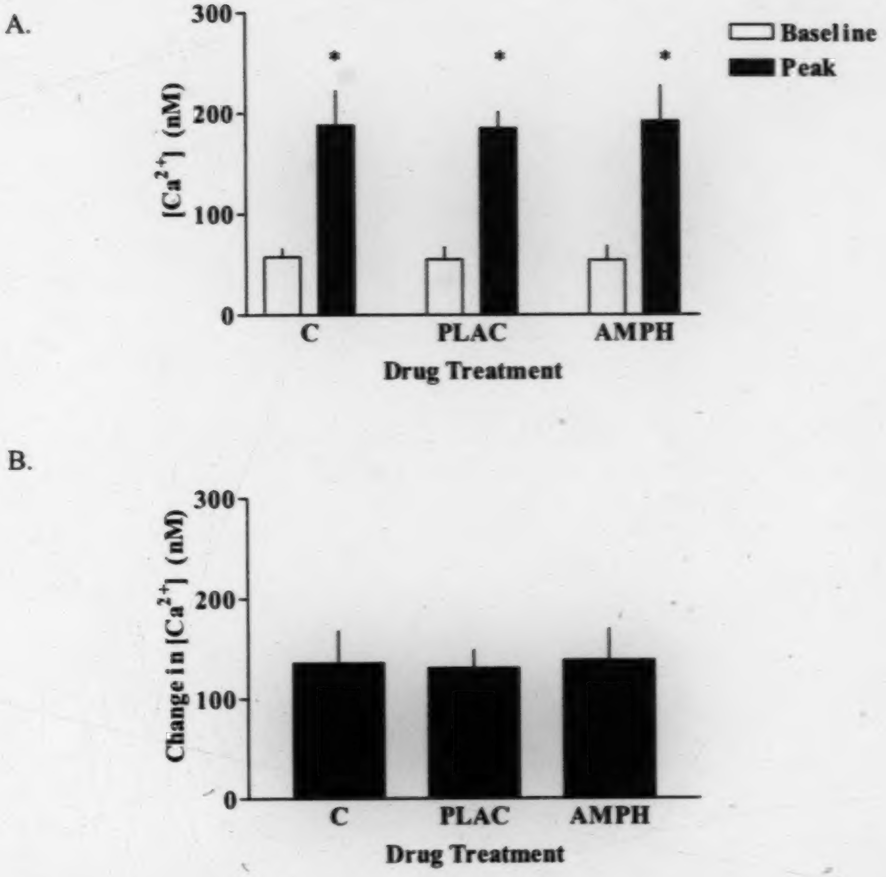

Figure 26 - Effects of *In Vivo* Dextroamphetamine on Thrombin-Induced Changes in Platelet $[Ca^{2+}]$

C controls (n = 12), PLAC placebo (n = 6), AMPH dextroamphetamine (n = 11), nM nanomolar, error bars represent standard error of mean. Measurements at time = 0 (C) or at time = 3.5 hours after administration of 25 mg dextroamphetamine or placebo. * significant increase in baseline to peak (F=128.040, df=1.00, $p < .001$). No significant differences were found between groups in basal, peak or change in $[Ca^{2+}]$ induced by .024 U/mL thrombin. Basal $[Ca^{2+}]$ (nM, means ± SEM): 57.3 ± 2.4 (C), 54.8 ± 5.1 (PLAC), 54.1 ± 4.2 (AMPH). Peak $[Ca^{2+}]$ (nM, means ± SEM): 188.0 ± 10.1 (C), 185.0 ± 6.8 (PLAC), 192.3 ± 10.8 (AMPH). Change in $[Ca^{2+}]$ (nM, means ± SEM): 135.3 ± 9.3 (C), 130.3 ± 7.4 (PLAC), 138.2 ± 9.4. (AMPH).

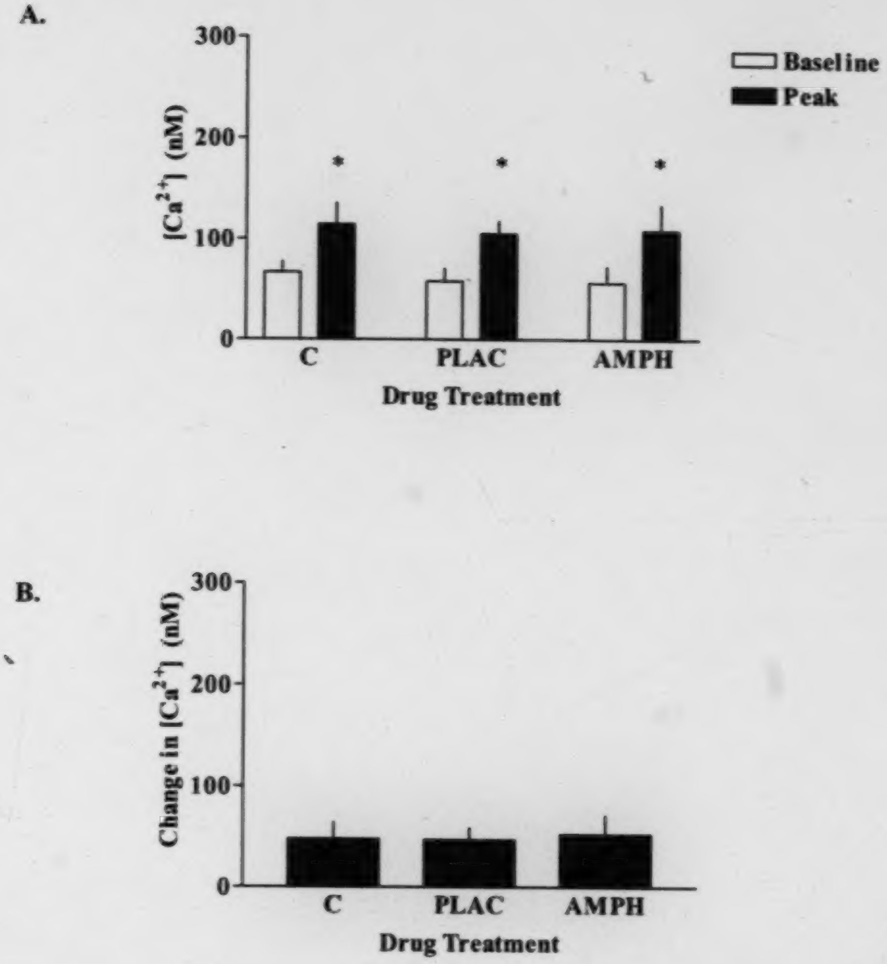

Figure 27 - Effects of *In Vivo* Dextroamphetamine on 5-HT-Induced Changes in Platelet $[Ca^{2+}]$

C controls (n = 12), PLAC placebo (n = 6), AMPH dextroamphetamine (n = 11), nM nanomolar, error bars represent standard error of mean. Measurements at time = 0 (C) or at time = 3.5 hours after administration of 25 mg dextroamphetamine or placebo. * significant increase in baseline to peak (F=95.776, df=1.00, $p < .003$). No significant differences were found between groups in basal, peak or change in $[Ca^{2+}]$ induced by 10 μM 5-HT. Basal $[Ca^{2+}]$ (nM, means ± SEM): 66.5 ± 3.0 (C), 57.9 ± 5.1 (PLAC), 56.3 ± 5.2 (AMPH). Peak $[Ca^{2+}]$ (nM, means ± SEM): 114.4 ± 6.1 (C), 105.0 ± 5.1 (PLAC), 108.7 ± 8.0 (AMPH). Change in $[Ca^{2+}]$ (nM, means ± SEM): 47.9 ± 4.7 (C), 47.1 ± 4.6 (PLAC), 52.4 ± 6.1 (AMPH).

3.4 Effects of *In Vitro* Dextroamphetamine, Lithium Chloride, Sodium Valproate, and Carbamazepine on Platelet Ca^{2+} Concentrations

There were no significant differences between replicate samples in any of the analyses, and thus replicate samples were averaged for each treatment group and graphed (Figures 28, 29).

Thrombin induced a significant increase in platelet Ca^{2+} concentration in all groups (F=170.657, df=1.00, $p < .001$), as expected (Figure 28). In samples stimulated with thrombin, a significant overall effect of treatment (F=21.764, df=2.548, $p < .001$), and on the interaction of treatment by agonist effect (F=42.239, df=1.772, $p < .001$) was found (Figure 28). Subsequent Tukey post-hoc analysis revealed that basal Ca^{2+} concentrations were significantly higher in all drug-treated groups compared to untreated samples. Basal $[Ca^{2+}]$ (nM, means ± SEM): 58.2 ± 2.3 for controls; 76.4 ± 3.1, $p < .002$ for lithium; 82.7 ± 3.4, $p < .001$ for valproate; 84.8 ± 3.3, $p < .001$ for carbamazepine; and 86.8 ± 3.9, $p < .001$ for dextroamphetamine.

Peak and change in Ca^{2+} concentrations were similar in all groups except in the carbamazepine-treated group, in which there was a significantly increased peak (277.1 ± 19.9 nM, $p < .002$) and change (191.9 ± 19.6 nM, $p < .046$) in Ca^{2+} concentration compared to controls (peak 195.8 ± 12.2 nM; change 142.0 ± 10.8 nM). Peak $[Ca^{2+}]$ (nM, means ± SEM) were 201.0 ± 9.4 for lithium; 193.1 ± 12.3 for valproate; and 206.0 ± 8.3 for dextroamphetamine. Change in $[Ca^{2+}]$ (nM, means ± SEM) were 124.3 ± 10.1 for lithium; 110.5 ± 10.5 for valproate; and 119.0 ± 7.7 for dextroamphetamine. Thrombin-induced peak Ca^{2+} concentrations were also significantly higher in the carbamazepine group compared with lithium ($p < .004$), valproate ($p < .002$) and dextroamphetamine ($p < .016$) samples. Change in [Ca^{2+}] induced by thrombin were also significantly higher in the carbamazepine group when compared with lithium ($p < .007$), valproate ($p < .001$) and dextroamphetamine ($p < .008$) treated samples.

5-HT induced a significant increase in platelet Ca^{2+} concentration in all groups (F=38.684, df=1.00, $p < .004$), as expected (Figure 29). In samples stimulated with 5-HT, a significant overall effect of treatment (F=26.343, df=2.092, $p < .001$) and on the interaction of treatment by agonist effect (F=33.639, df=2.552, $p < .001$) was found (Figure 29). Subsequent Tukey post-hoc analysis revealed that basal Ca^{2+} concentrations are higher in drug-treated groups which is significant for valproate, carbamazepine and dextroamphetamine. There was a trend towards significance in the lithium-treated groups. Basal [Ca^{2+}] (nM, means ± SEM): 67.0 ± 2.8 for controls; 75.0 ± 2.4, $p = .346$ for lithium; 85.0 ± 3.5, $p< .002$ for valproate; 84.7 ± 2.9, $p < .002$ for carbamazepine; 85.9 ± 4.3, $p < .003$ for dextroamphetamine.

Peak and change in Ca^{2+} concentrations are similar in all groups except the carbamazepine-treated group in which there is a significantly increased peak (153.0 ± 8.2 nM, $p < .003$) and trend to significant change (68.3 ± 6.0 nM, $p < 0.54$) in Ca^{2+} concentration compared to controls (peak 115.4 ± 5.7 nM; change 48.4 ± 4.3 nM). Peak [Ca^{2+}] (nM, means ± SEM) were 116.8 ± 5.3 for lithium; 121.5 ± 5.2 for valproate; and 122.2 ± 12.2 for dextroamphetamine. Change in [Ca^{2+}] (nM, means ± SEM) were 41.9 ± 5.1 for lithium; 36.5 ± 4.1 for valproate; 36.3 ± 8.5 for dextroamphetamine. 5-HT-induced peak Ca^{2+} concentrations were also significantly higher in the carbamazepine group when compared with lithium ($p < .007$) and valproate ($p < .018$) samples; and nearly significant when compared with dextroamphetamine ($p < .054$) samples. Change in [Ca^{2+}] induced by 5-HT were also significantly higher in the carbamazepine group when compared with lithium ($p < .01$, valproate ($p < .002$) and dextroamphetamine ($p < .004$) samples.

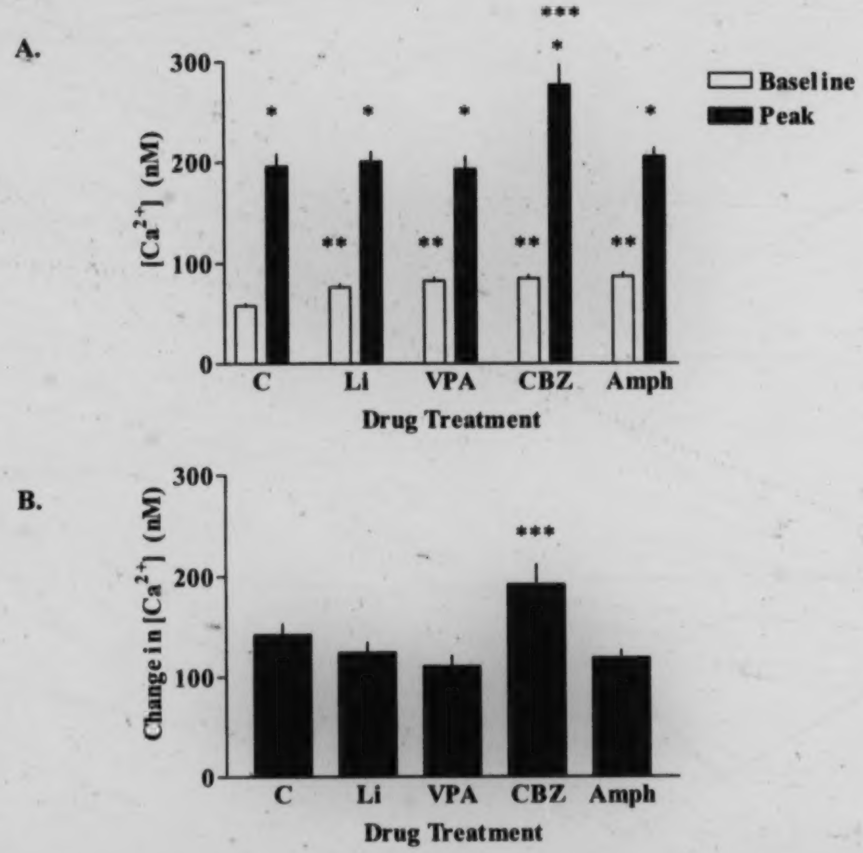

Figure 28 - Effects of *In Vitro* Lithium Chloride, Sodium Valproate, Carbamazepine or Dextroamphetamine on Thrombin-Induced Changes in Platelet $[\mathrm{Ca}^{2+}]$

C controls (n = 13), Li lithium chloride (n = 10), VPA sodium valproate (n = 11), CBZ carbamazepine (n=11), Amph dextroamphetamine (n = 7), nM nanomolar, error bars represent standard error of mean. Measurements after incubation with 1.0 μM lithium chloride, 600 μM sodium valproate, 40 μM carbamazepine, 50 ng/mL dextroamphetamine or no drug for 40 minutes at room temperature. * significant increase in baseline to peak (F=170.657, df=1.00, $p < .001$). ** significant increase in basal $[\mathrm{Ca}^{2+}]$ after treatment with lithium chloride ($p < .002$), sodium valproate ($p < .001$), carbamazepine ($p < .001$) or dextroamphetamine ($p < .001$) *** treatment with carbamazepine significantly increased peak $[\mathrm{Ca}^{2+}]$ ($p < .002$) and change in $[\mathrm{Ca}^{2+}]$ ($p < .046$). Basal $[\mathrm{Ca}^{2+}]$ (nM, means ± SEM): 58.2 ± 2.3 (C), 76.4 ± 3.1 (Li), 82.7 ± 3.4 (VPA), 84.8 ± 3.3 (CBZ), 86.8 ± 3.9 (Amph). Peak $[\mathrm{Ca}^{2+}]$ induced by .024 U/mL thrombin (nM, means ± SEM): 195.8 ± 12.2 (C), 201.0 ± 9.4 (Li), 193.1 ± 12.3 (VPA), 277.1 ± 19.9 (CBZ), 206.0 ± 8.3 (Amph). Change in $[\mathrm{Ca}^{2+}]$ induced by .024 U/mL thrombin (nM, means ± SEM): 142.0 ± 10.8 (C), 124.3 ±10.1 (Li), 110.5 ± 10.5 (VPA), 191.9 ± SEM = 19.6 (CBZ), 119.0 ± 7.0 (Amph).

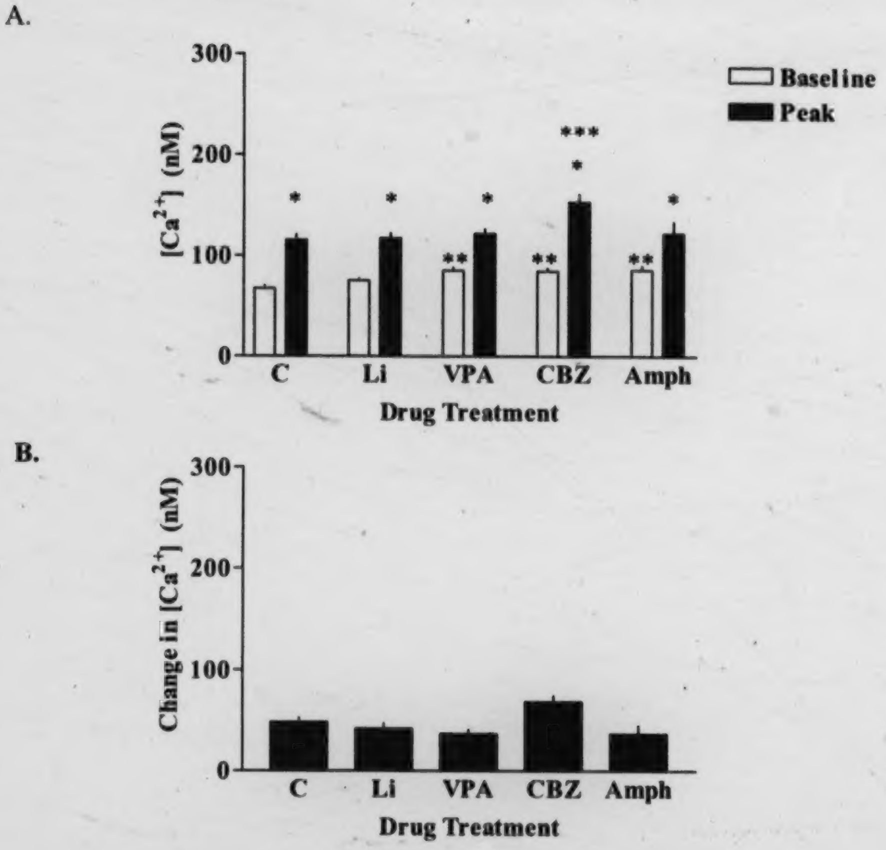

Figure 29 - Effects of *In Vitro* Lithium Chloride, Sodium Valproate, Carbamazepine or Dextroamphetamine on 5-HT-Induced Changes in Platelet $[Ca^{2+}]$

C controls (n = 13), Li lithium chloride (n = 10), VPA sodium valproate (n = 11), CBZ carbamazepine (n=11), Amph dextroamphetamine (n = 7), nM nanomolar, error bars represent standard error of mean. Measurements after incubation with 1.0 μM lithium chloride, 600 μM sodium valproate, 40 μM carbamazepine, 50 ng/mL dextroamphetamine or no drug for 40 minutes at room temperature. * significant increase in baseline to peak (F=38.684, df=1.00, $p < .004$). ** significant increase in basal $[Ca^{2+}]$ after treatment with sodium valproate ($p < .002$), carbamazepine ($p < .002$) or dextroamphetamine ($p < .003$) *** treatment with carbamazepine significantly increased peak $[Ca^{2+}]$ ($p < .003$). Basal $[Ca^{2+}]$ (nM, means ± SEM): 67.0 ± 2.8 (C), 75.0 ± 2.4 (Li), 85.0 ± 3.5 (VPA), 84.7 ± 2.9 (CBZ), 85.9 ± 4.3 (Amph). Peak $[Ca^{2+}]$ induced by 10 μM 5-HT (nM, means ± SEM): 115.4 ± 5.7 (C), 116.8 ± 5.3 (Li), 121.5 ± 5.2 (VPA), 153.0 ± 8.2 (CBZ), 122.2 ± 12.2 (Amph). Change in $[Ca^{2+}]$ induced by 10 μM 5-HT (nM, means ± SEM): 48.4 ± 4.3 (C), 41.9 ± 5.1 (Li), 36.5 ± 4.1 (VPA), 68.3 ± 6.0 (CBZ), 36.3 ± 8.5 (Amph).

CHAPTER 4 – DISCUSSION

4.1 *In Vivo* Dextroamphetamine Does Not Change Basal or Agonist-Induced Platelet Ca^{2+} Concentrations While *In Vitro* Dextroamphetamine Increases Basal Platelet Ca^{2+} Concentrations

Dextroamphetamine is often used as a psychological model of mania in the study of bipolar disorder, as this drug induces a manic-like syndrome which includes symptoms of euphoria, racing thoughts, increased talkativeness, goal-directedness, self-esteem, distractibility, and decreased need for sleep (Mamelak 1978, Jacobs and Silverstone 1986). There is also evidence that dextroamphetamine is a good biological model of mania as it has been found to induce biological effects similar to those found in manic patients: increased heart rate, blood pressure (Jacobs and Silverstone 1986), catecholamine activity (Diehl and Gershon 1992) and cerebral metabolism (Vollenweider et al. 1998). The well-defined involvement of the PI-cycle in bipolar disorder has lead to the investigation of the effects of dextroamphetamine on the PI-cycle. *In vivo* MRS studies have revealed that dextroamphetamine increases phosphomonoesters (PMEs) (a measure of inositol phosphates) in healthy volunteers that is potentiated after one week of lithium treatment (Silverstone et al. 2000). Furthermore, dextroamphetamine produced an increase in PMEs in healthy volunteers, an effect blocked in euthymic bipolar patients medicated either with chronic lithium or valproate (Silverstone et al. 2000, unpublished data). An earlier study showed that dextroamphetamine also caused an increase in *myo*-inositol in rat brain (Barkai 1981). Together these findings suggest that dextroamphetamine activates the PI-cycle.

To my knowledge, the study reported in this thesis is the first one that investigates the effects of dextroamphetamine on platelet Ca^{2+} concentrations in healthy human subjects. Since an enhanced thrombin- (Dubovksy et al. 1989, Tan et al. 1995) and 5-HT- (Berk et al. 1995, Okamoto et al. 1995, Yamawaki et al. 1998) induced Ca^{2+} response is associated with mania, it is reasonable to expect that the manic-like syndrome induced by dextroamphetamine will be accompanied by a similar enhancement of agonist-induced platelet Ca^{2+} responses, as stated in the working hypothesis. The results reported in this study do not support such a theory, since no differences were found after *in vivo* dextroamphetamine administration on any measure of platelet Ca^{2+}. Further, *in vitro* dextroamphetamine did not affect agonist-induced platelet Ca^{2+} responses either.

However, these results are in agreement with previous studies examining the effects of dextroamphetamine-related *in vivo* variations on platelet $[Ca^{2+}]$. A similar double-blind placebo-controlled crossover study with carmoxirole, a dopamine (D2) receptor agonist (which would be expected to be similar to dextroamphetamine in its biological effects), given once daily for a week, also reported no changes in platelet intracellular Ca^{2+} responses in healthy subjects (Lijnen et al. 1993). Another study reported that agonist-induced platelet $[Ca^{2+}]$ is not altered by the effects of smoking or physical activity which included increased blood pressure, heart rate, peripheral NE [effects also produced by dextroamphetamine (Goldstein et al. 1983)], E and vasopressin (VP) (Mooser et al 1988). Finally, *in vivo* lithium administration for one, two, three or four weeks to healthy male subjects had no effects on basal, thrombin- or 5-HT-induced platelet calcium concentrations (Kusumi et al 1994a). This last study indicates that lithium, a centrally acting agent like amphetamine, does not affect peripheral blood cell Ca^{2+} concentrations. These studies indicate that agents, such as dextroamphetamine, which cross the blood-brain barrier, affect the PI-cycle in the central nervous system, may not necessarily affect peripheral blood cell Ca^{2+}. However, several factors should be considered in the interpretation of these results concerning the *in vivo* model of mania.

As with any research model, there are limitations that must be considered in the dextroamphetamine-induced model of mania. While the evidence discussed above does suggest that dextroamphetamine models both psychological and biological characteristics of mania, whether or not dextroamphetamine affects the same pathways on a molecular level that are abnormal in bipolar mania is still disputed. If this is the case, it could explain why no differences in platelet Ca^{2+} concentrations were found after *in vivo* administration with dextroamphetamine in this study. There is evidence suggesting that the PI-cycle may not be involved in all of the effects induced by dextroamphetamine. Indeed, dextroamphetamine-induced burst-firing potentials in snail neurons that occurred via the activation of the cAMP pathway were unaltered by PKC inhibition (Chen and Tsai 1997). Moreover, while some studies have shown that lithium attenuated dextroamphetamine-induced euphoria in depressed patients (Van Kaman and Murphy 1975) and dextroamphetamine-induced hyperactivity in rats (Bergen 1985), others have shown that lithium did not attenuate the psychological effects induced by dextroamphetamine in healthy volunteers (Silverstone et al. 1998) nor does it attenuate dextroamphetamine-induced hyperactivity in rats (Fester et al. 1982, Caponize and Moore 1990), suggesting that the mood stabilization produced by lithium occurs via a mechanism different to that involved in the manic-like syndrome induced by dextroamphetamine.

In view of these findings, the lack of effect of *in vivo* dextroamphetamine on platelet Ca^{2+} reported in this thesis may be explained by the following limitations inherent to the dextroamphetamine model of mania. Firstly, the duration of the manic-like syndrome produced by dextroamphetamine is short, lasting from 2-4 hours, with peak psychological effects occurring around 2 hours compared to bipolar mania which typically lasts for several days. In addition, the bipolar patients from whom enhanced agonist-induced platelet Ca^{2+} responses have been found may have experienced repeated episodes of mania, which could subsequently lead to chronic alterations in their mechanisms for maintaining Ca^{2+} homeostasis. Thus, the acute administration of dextroamphetamine may be unable to mimic all chronic biological changes present in bipolar disorder. Secondly, the symptoms produced by dextroamphetamine at the doses used in research (~20-30 mg or 0.3-0.4 mg/kg), are usually much milder than those actually experienced in mania and therefore may not be strong enough to produce some measurable biological effects. For example, healthy volunteers who have acutely received such a dose of dextroamphetamine, do not become involved in the high-risk activities which alter the day-to-day functioning of manic patients. Thirdly, platelet Ca^{2+} abnormalities found in bipolar manic patients may not be related to their state, but may actually be a trait of the disorder. This is supported by the fact that the increase in platelet $[Ca^{2+}]$ induced by both thrombin (Dubovsky et al. 1989, 1991, Kusumi et al. 1992) and 5-HT (Kusumi et al. 1994b, Berk et al. 1995) is also enhanced in bipolar depression. Although several studies report normal agonist-induced $[Ca^{2+}]$ in bipolar patients in the euthymic state (Bothwell et al. 1994, Berk et al. 1994, 1995, Dubovsky et al. 1989, 1991, Okamoto et al. 1995), an enhanced agonist-induced $[Ca^{2+}]$ may be masked by the medication the patients were taking in these studies (lithium alone or lithium in combination with other psychiatric drugs). Thus the mechanism by which agonist-induced platelet [Ca^{2+}] is enhanced in bipolar disorder may be related to a genetic trait of these patients which, for example, could be linked to an abnormal production of second messengers or Ca^{2+} channels. The *in vivo* dextroamphetamine model of mania will not likely model several of these effects at the molecular level.

Finally, the use of platelets must be considered: the mechanisms by which dextroamphetamine acts on platelets may not model the effects it induces in the brain. In the brain dextroamphetamine increases the activation of DA and NE receptors, and to some extent 5-HT. The mechanisms in which dextroamphetamine acts on central pre-synaptic neurons are by blocking the re-uptake of biogenic amines, stimulating their release, and at high concentrations, weakly blocking MAO enzymes. Dextroamphetamine also acts on post-synaptic neurons by directly stimulating these receptors due to its structural similarity to the biogenic amines (Seiden et al. 1993, Kuczenski and Segal 1994). These are effects that can lead to the stimulation of receptors linked to the PI-cycle in the brain and thus activation of the PI-cycle.

Any *in vivo* effects of dextroamphetamine administration on platelets may be modulated by a parallel increase in neurotransmitter levels in the plasma or by a direct contact of amphetamine with platelets. However, the concentrations of dextroamphetamine and of any neurotransmitters released into the periphery may not reach high enough levels for periods of time long enough to have any observable effects on platelet Ca^{2+} concentrations. Of course, the effective dose of dextroamphetamine for neurons is probably different for platelets in producing similar biological changes. Also, DA, NE and 5-HT are relatively weak agonists of platelet activation (Siess 1989, Scrutton 1993), while the direct effects of dextroamphetamine on platelet Ca^{2+} concentrations have not been extensively studied. Thus, to date the mechanisms by which dextroamphetamine could potentially be acting on platelet signal transduction pathways can only be speculated.

In this study, the plasma concentration of dextroamphetamine reached its maximum at approximately 3 hours (Asghar et al. 2000, unpublished data), the time of peak plasma levels reported in previous literature (Kupietx et al. 1985), confirming that platelets, which were collected 3.5 hours after dextroamphetamine administration, would have had some exposure to dextroamphetamine in the plasma. However, once the platelets were isolated from blood plasma, the drug was no longer present in the buffers used during the experimental protocol, which lasted about 3.5 hours. Thus, any short-lived effects of dextroamphetamine may have been lost. That an acute effect of dextroamphetamine on platelets does occur is supported by the finding that 50 ng/mL dextroamphetamine incubated *in vitro* with platelets for 40 minutes caused an increase in basal platelet Ca^{2+} concentrations. Thus, while dextroamphetamine may have the ability to alter platelet Ca^{2+} concentrations, this effect may not be detectable at the concentrations and times of exposure used in the present *in vivo* experiment. The mechanism by which *in vitro* dextroamphetamine increases basal platelet Ca^{2+} concentrations may be due to a direct activation of platelet receptors or an indirect increase in plasma catecholamines, both effects potentially leading to PI-cycle activation of increases in cytosolic $[Ca^{2+}]$, as discussed previously. Further investigation of the direct effects of dextroamphetamine on platelets will help to reveal the pathways involved, and the clinical relevance, of these findings.

4.2 *In Vitro* Effects of Mood Stabilizers on Platelet Ca^{2+} Concentrations

4.2.1 Lithium Chloride, Sodium Valproate and Carbamazepine Increase Basal Ca^{2+} Concentrations in Platelets

Several studies have reported normal basal platelet Ca^{2+} concentrations in unmedicated manic (Berk et al. 1994, 1995, Okamoto et al. 1995), depressed (Kusumi et al. 1992, 1994b, Berk et al. 1994, 1995) and medicated euthymic (Bothwell et al. 1994, Dubovsky et al. 1989, 1991, Okamoto et al. 1995) bipolar patients, suggesting that basal Ca^{2+} is not affected by the illness, mood state or mood stabilizers. Surprisingly, the results from this study showed that all the mood stabilizers used (lithium, valproate and carbamazepine) increased basal platelet Ca^{2+} concentrations and did not attenuate agonist-induced Ca^{2+} responses. This is opposite to the predictions made from our working hypothesis in which no changes in basal Ca^{2+}, and a decrease in the agonist-induced Ca^{2+} responses, were expected.

To understand this contradiction, it is useful to consider the inositol-depletion hypothesis of lithium and the effects of valproate and carbamazepine on the PI-cycle. The inhibition of IMPase by lithium causes a decrease in *myo*-inositol concentrations (Allison and Stewart 1971, Sherman et al. 1981, Sherman et al. 1985, Hirvonen 1991, Hirvonen and Savolainen 1991, Sun et al. 1992, O'Donnell et al. 2000) which in turn leads to an increase in the concentrations of I-1-Ps (Allison et al. 1976, Sherman et al. 1981, Sherman et al. 1985, Savolainen et al. 1990, Hirvonen 1991, Preece et al. 1992, Lubrich et al. 1997). The build-up of I-1-Ps may be accompanied by a subsequent increase in IP_3 due to the cessation of the cycle. Lithium (Whitworth et al. 1990, Dixon et al. 1992, Lee et al. 1992, Sun et al. 1992, Ishima et al. 1993, Dixon et al. 1994) and valproate (Dixon and Hokin 1997) have both been shown to increase IP_3 concentrations. An increase in IP_3 would likely lead to an increase in cytosolic [Ca^{2+}] via IP_3- sensitive calcium regulation. Therefore, it is conceivable that while the chronic effects of these drugs are to attenuate PI-cycle activation by blocking steps in and depleting components of the pathway, the acute effects result in a transient imbalance in second messengers, e.g. IP_3, that signal an increase in cytosolic [Ca^{2+}]. Thus, a short-lived increase in intracellular Ca^{2+} may occur. Therefore, the acute incubation of mood stabilizers with platelets (40 minutes) in this study may have produced a transient imbalance, resulting in an increase in intracellular Ca^{2+}. The attenuating effects expected of these drugs may only be produced with chronic administration. In fact, there are several studies which report differences in the acute and chronic effects of mood stabilizers (Renshaw et al. 1996, Lenox et al. 1992, 1996, Song and Jope 1992, Ishima et al. 1993, Manji et al. 1993, 1996b, Bebchuk et al. 1998, Watson et al. 1998, Wang and Friedman 1999). Thus, differences in the results reported between the studies reviewed in chapter 1 (Table 1 and 2) may be explained by variations in treatment length. This reasoning can also be applied to previous findings in similar studies to explain inconsistent results between studies.

Effects of mood stabilizers on platelet Ca^{2+} have been previously reported based on *in vitro* incubation studies. Incubation of platelets with 1 mM lithium for 60 minutes resulted in a decreased basal platelet $^{45}Ca^{2+}$-uptake in manic patients, depressed patients and healthy controls (Berk et al. 1996). The differences between this finding with those from the present study are probably due to one of three key methodological differences. Firstly, a 1000x lower dose of lithium was used in the present experiment (1 μM versus 1 mM). While neither of these doses is within the range of therapeutic plasma levels (0.6

to 1.2 mM), the dose of lithium used in the study reported in this thesis may be a closer representation of brain levels which have been shown to be much less than those in plasma (Ghoshdastidar et al. 1989, Kushnir et al. 1993). It likely that at very high doses, lithium causes cellular toxicity which may result in a decreased cellular responsiveness not related to its therapeutic mechanisms of action. Secondly, in this study, platelets were incubated for 40 minutes at room temperature, while the incubation conditions for the former study (Berk et al. 1996) were 60 minutes at 37°C, suggesting that different incubation temperatures might lead to different results. In this study, platelets were incubated with the drugs at room temperature, because excessive dye-leakage occurred when cells were incubated at 37°C. However, samples were pre-warmed for 4 minutes and basal and agonist-induced Ca^{2+} concentrations were measured at 37°C in this study. Thirdly, the findings in the present study are reported as basal Ca^{2+} concentrations, while the former studies actually measured basal Ca^{2+} *influx*. Thus, comparing these two findings, lithium may decrease the amount of Ca^{2+} coming into the cell, while the actual Ca^{2+} concentration may be affected differently.

In another study, *in vitro* incubation of lymphocytes with similar concentrations of carbamazepine as used in this study (40 μM) resulted in a decrease in basal lymphocyte Ca^{2+} concentrations in manic and depressed patients, but not in healthy controls (Dubovsky et al. 1994). These findings suggest that a decrease in basal Ca^{2+} concentrations will be seen only in patients in which the basal Ca^{2+} levels may be abnormally elevated. However, the methods employed in this study were again different to the present study: (i) the effects of carbamazepine on lymphocytes and platelets are likely to be different and (ii) the study by Dubovsky et al. (1994) involved the incubation of lymphocytes with carbamazepine for 18 hours, so longer-term effects were examined in contrast to the short-term effects studied in the present study (40 minutes). Again, chronic versus acute effects of carbamazepine may be the key to the differences in results obtained: chronic carbamazepine decreases, while acute carbamazepine increases platelet Ca^{2+} concentrations. For instance, after 18 hours, compensatory mechanisms may have taken place, while after only 40 minutes these processes may have not occurred.

Another study similar to the present experiment shows that *in vitro* incubation of platelets with lithium, valproate and carbamazepine had no effect on basal, thrombin- or 5-HT-induced Ca^{2+} concentrations (Kusumi et al. 1994a). However, in addition to the differences in the length of drug treatment, this study illustrates that differences in results can often be explained by several methodological differences in platelet Ca^{2+} measurements, which are summarized in Table 7.

Table 7 - Effects of Mood Stabilizers on Platelet $[Ca^{2+}]$: Methodological Considerations

	This Study	**Kusumi et al. 1994a**
Major Findings	↑ basal $[Ca^{2+}]$ with lithium, valproate and carbamazepine ↑ thrombin- and 5-HT-induced $[Ca^{2+}]$	**no change** in basal, thrombin- or 5-HT-induced $[Ca^{2+}]$ with lithium, valproate or carbamazepine
Basal $[Ca^{2+}]$ (no drug) (nM, ± SEM)	57.3 ± 2.4 66.5 ± 3 58.2 ± 2.3 67.0 ± 2.8	75.7 ± 7.3
Drug concentrations	Lithium 1.0 µM Valproate 600 µM Carbamazepine 40 µM	Lithium 10 mM Valproate 100 µM Carbamazepine 100 µM
Dye Incubation Conditions	30 minutes 37°C HEPES buffer with citrate 3 µM Fura-2AM	15 minutes 37°C PRP with citrate 4 µM Fura-2AM
Agonist Concentrations	Thrombin 0.024 U/mL 5-HT 10 µM	Thrombin 1.0 U/mL 5-HT 0.3 µM or 10 µM
Drug Incubation Conditions	40 minutes, room temperature in platelet suspensions, directly followed by measurements	1 or 4 hours, 37°C in PRP, followed by centrifugation, re-suspension, measurements
Drug Present in External Media During Measurements and agonist stimulation?	Yes	No
Platelet Concentrations	3.5 x 10^8 cells/mL (incubation) 1.0 x 10^8 cells/mL (measurements)	[PRP] variable (incubation) 1 x 10^8 cells/mL (measurements)
Approximate Average $[Ca^{2+}]$ Determined (nM)	Basal 60 Thrombin 190-220 5-HT 90-120	Basal 70 Thrombin 1600 5-HT (0.3 µM) 150 5-HT (10 µM) 200

Of particular importance is the fact that the drugs are absent from the external media in the former study by Kusumi et al. (1994a) since the cells are re-pelleted and re-suspended in drug-free solutions after the drug incubations. Thus, unless the drugs have induced long-term cellular alterations (which are unlikely in less than 4 hours), there is no way for them to alter the cell unless they are present during the measurements. Differences could also be related to the concentration of the platelets in the incubation media, which were 3.5 x 10^8 cells/mL in the current study, while they will be variable in the PRP incubated in the former study. As discussed in chapter 2, differences in platelet concentration during incubation times can lead to differences in the concentration of agonist-induced platelet [Ca^{2+}] (Figure 15).

Thus, these novel findings that mood stabilizers increase basal platelet Ca^{2+} concentration are interesting and require further study to reveal their relevance. With regard to similar measurements, the lack of reproducibility of trends in platelet Ca^{2+} concentrations is probably due to either methodological differences between studies or to differences in the acute and chronic effects of the drugs.

4.2.2 Carbamazepine Enhances the Increase in Platelet Calcium Ions Induced by Thrombin and 5-HT

Carbamazepine *enhanced* the agonist-induced increase in [Ca^{2+}] and did not, as expected, *attenuate* Ca^{2+} responses. This result may be explained by the fact that the acute effects of carbamazepine may be different than the chronic effects it induces, as discussed in the previous section.

To my knowledge there are only two studies that have reported the effects of *in vitro* carbamazepine on peripheral blood cells. In one study carbamazepine was incubated with lymphocytes for 18 hours from manic and depressed bipolar patients and healthy controls (Dubovsky et al. 1994). In that study, carbamazepine decreased PHG-induced [Ca^{2+}] in both patient groups, but not in the healthy controls. In another study, carbamazepine was incubated with PRP from healthy controls and no effect was found on thrombin- or 5-HT-induced Ca^{2+} responses (Kusumi et al. 1994a). As discussed in the previous section; (i) the chronic effects of carbamazepine may attenuate agonist-induced platelet [Ca^{2+}], while its acute affects increase agonist-induced platelet [Ca^{2+}] following a transient imbalance of the PI-cycle; and (ii) the methodological differences among these three studies make comparison of the results problematic.

The mechanism by which carbamazepine enhances agonist-induced platelet [Ca^{2+}] in the present study is unclear. It is generally accepted that carbamazepine, at therapeutic doses, is both a Na^+ channel blocker (Davies 1995, Meldrum 1996) and a Ca^{2+} channel blocker (Winkel and Lux 1987, Walden et al. 1992, 1993, Schirrmacher et al. 1993, Yoshimura et al. 1995, Yamaji et al. 1996, Ambrosio et al. 1999). There are also several reports from animal studies showing that carbamazepine attenuates PI-cycle activation with a decrease in I-1-P formation (Vadnal and Bazan 1988, McDermott and Logan 1989, Biber et al. 1996), membrane phospholipid formation (Wei and Wang 1987) and PKC activation (Jensen and Mork 1997). Common to the above effects is an attenuation of cellular excitability and signal transduction. The increase in cellular Ca^{2+} found in this study then is likely due to the key differences between these studies with the present study. The above studies report either the chronic effects of carbamazepine or its effects on neuronal tissue, while this study reports the acute effects of carbamazepine on non-neuronal tissue. Carbamazepine has been shown to inhibit voltage-gated ion channels (Na^+ and Ca^{2+}) in neurons, while these channels probably do not exist in platelets (Rink and Sage 1990). The major types of Ca^{2+} channels involved in platelet Ca^{2+} homeostasis are receptor-operated-, store-regulated- and second-messenger-operated (Sage et al. 1993). Hence, the combination of cellular effects induced by carbamazepine may manifest differently in different cell types, and with different lengths of treatment, due to the complexity of interactions between its targeted pathways.

The question still remains as to why carbamazepine enhanced agonist-induced platelet Ca^{2+} responses, while lithium and valproate did not. The reason may be due to differences in the mechanisms of carbamazepine compared with lithium and valproate. Carbamazepine and valproate both reduce voltage-gated Ca^{2+} currents, however with different potency and channel selectivity (Meldrum 1996), while lithium does not appear to bind directly to Ca^{2+} channels (Silverstone and Grahame-Smith 1991). With regard to the PI-cycle, carbamazepine stimulated IMPase activity isolated from bovine brain, while valproate had no effect, in contrast to lithium, which inhibited IMPase (Vadnal and Parthasarathy 1995). With regard to platelets, carbamazepine inhibited 5-HT uptake in rat and human platelets (and rat synaptosomes) while lithium and valproate did not (Southam et al. 1998). Thus, while it is still likely that the therapeutic actions of mood stabilizers may ultimately lead to a common attenuation of cellular activity in bipolar disorder, each of them may do so by affecting different pathways. In this study, carbamazepine affects a pathway involved in thrombin- and 5-HT-induced calcium influx in platelets. Lithium and valproate may affect a different part of the same pathway or a different pathway altogether, which could explain the differences between these three mood stabilizers.

4.3 Methodological Considerations

The experimental manipulations discussed in Chapter 2 show that the results obtained are strongly dependent on the specific aspects of the protocol used. In comparing the values for $[Ca^{2+}]$ between studies listed in Tables 5 and 6, it is obvious that a large range exists between studies as small variations in platelet handling techniques, dye loading conditions, temperatures of experiments, concentration of cells and doses of agonists which all can lead to large differences in the actual determination of $[Ca^{2+}]$.

When considering the values for Ca^{2+} concentrations reported in Tables 5 and 6, it is clear that several of these values are much higher than the K_d of Fura-2 (~224 nM). Therefore values between 500 and 1000 nM that are reported for thrombin-induced Ca^{2+} concentrations are probably not very accurate representations of the actual concentrations of Ca^{2+} (Dubovsky et al. 1989, 1991, Eckert et al. 1993, Bothwell et al. 1994, Kusumi et al 1994a, Adunsky et al. 1995). This problem could be overcome by the use of much lower concentrations of thrombin.

In general, differences in type and dose of agonists, platelet concentrations and incubation conditions therefore make it difficult to compare results between studies. It is thus more useful to compare general trends within studies. Methodological differences must always be considered as potentially confounding variables when interpreting results in these studies.

4.4 Use of Platelets in Psychiatric Research

The validity of using the platelet as a model system in psychiatric research is based on similarities in the cellular machinery that exists between platelets and neurons. Although not a perfect model, the platelet is a useful tool to study second messenger pathways in a clinical setting, which is otherwise limited. While the findings from peripheral blood platelets may not be directly extrapolated to brain studies, they do give an overall indication of which pathways may be altered in an illness or by medications. In research on bipolar disorder, peripheral measures such as IPs, PKC and PIP_2 give support to the involvement of the PI-cycle in bipolar disorder, while peripheral measures of intracellular $[Ca^{2+}]$ support the involvement of Ca^{2+}. Thus, platelet models are useful in the clinical setting to gather general information about signal transduction pathways and, in conjunction with animal models, give clues about the molecular mechanisms involved within specific pathways.

CONCLUSIONS

There is abundance of evidence supporting the involvement of the PI-cycle in bipolar disorder and in the mechanisms of mood stabilizers. The clinical study of second messengers in bipolar disorder is currently limited to peripheral blood cells and *in vivo* MRS measures. MRS studies are limited to the measurement of *myo*-inositol and phosphomonoesters and currently require methodological improvements to produce accurate results. Peripheral blood cells are useful models to study a variety of second messenger pathways in the clinical setting. Elucidating the mechanisms by which (i) mood stabilizers produce their therapeutic effects in bipolar patients and (ii) dextroamphetamine induces a manic-like syndrome in healthy subjects, is useful in providing insight into the underlying pathophysiology of bipolar disorder.

The evidence from this study shows that the acute effects of both mood stabilizers (lithium, valproate and carbamazepine) and dextroamphetamine on platelets are to increase basal Ca^{2+} concentrations, and that carbamazepine additionally enhances both thrombin- and 5-HT-induced increases in platelet $[Ca^{2+}]$. It is suggested that these drugs produce these effects by causing a transient imbalance of the PI-cycle, which leads to accumulation of IP_3, the second messenger which increases cytosolic Ca^{2+}, and consequently a short-lived increase in intracellular Ca^{2+}. In testing this concept, it would be useful to replicate these experiments in a series of time points looking at both acute and chronic drug exposures. In determining the molecular mechanisms by which intracellular calcium is affected in this study could be assessed by (i) concurrently measuring IP_3, PKC and other second messengers which are linked to changes in cytosolic $[Ca^{2+}]$ in platelets and (ii) determining the involvement of calcium channels on these results by including Ca^{2+} channel blockers in these experiments. Furthermore, it would also be useful to carry out these experiments in neuronal tissues (*in vivo* animal models or cell cultures) as well as platelets to test for tissue-specific effects. In conclusion, the platelet Ca^{2+} measures described in this study give new insights into possible mechanisms of involvement of intracellular Ca^{2+} in bipolar disorder and warrant further investigation.

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Chemical	Manufacturer	Catalogue #
(+) Amphetamine Sulphate	Smith Kline and French Canada Ltd.	Lot # 87/175/15
Apyrase	Sigma Chemicals	A-6132
Calcium Calibration Buffer Kit with Magnesium # 1	Molecular Probes	C-3721
Calcium Chloride Dihydrate	Aldrich Chemical Company Inc.	22, 350-6
Calcium Sponge D70 (BAPTA dextran, 70, 000 MW anionic)	Molecular Probes	C-3041
Carbamazepine (5H-Dibenz[b,f]azepine-5-carboxamide)	Sigma Chemicals	C-4024
Citric Acid (monohydrate)	Sigma Chemicals	C-0706
D-(+) Glucose (dextrose) anhydrous	Sigma Chemicals	G-5767
DMSO (Dimethyl Sulfoxide) (anhydrous)	Fisher Scientific	BP231-1
EGTA (Ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) (tetrasodium salt)	Sigma Chemicals	E-8145
Fura-2, AM (acetoxy methylester) (cell permeant) (20 x 50 ug pkg)	Molecular Probes	F-1221
Fura-2, pentapotassium sait (cell impermeant)	Molecular Probes	F-1200
HEPES salt (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)(free acid)	Fisher Scientific	BP310-500
Lauryl Sulfate (sodium dodecyl sulphate, SDS) (sodium salt)	Sigma Chemicals	L-4509
Lithium Chloride (anhydrous)	Sigma Chemicals	L-0505
Magnesium Chloride (hexahydrate)	Fisher Scientific	M-33-500
Magnesium Sulphate (anhydrous)	Fisher Scientific	M-65
Manganese Chloride (tetrahydrate)	Sigma Chemicals	M-3634
Nitric Acid	Fisher Scientific	A-200-212
Pluronic F-127 (PF-127)	Sigma Chemicals	P-2443
Prostaglandin I_2 (prostacyclin)	Sigma Chemicals	P-6188
Serotonin (5-hydroxytryptamine) (5-HT) (creatinine sulphate complex)	Sigma Chemicals	H-7752
Sodium Chloride	Fisher Scientific	S-640-3

Sodium Citrate (trisodium salt: dihydrate)	Sigma Chemicals	S-4641
Sodium Phosphate Dibasic Anhydrous	Fisher Scientific	S374-500
Thrombin (from human plasma) 14 NIH units per vial	Sigma Chemicals	T-9010 (Lot # 85H9301)
TPEN (N,N,N′,N′-tetrakis(2-pyridyl-methyl)ethylenediamine	Sigma Chemicals	P-4413
Trizma Base 2-([hydroxymethyl]aminomethane	Fisher Scientific	T-1503
Trizma HCl 2-([hydroxymethyl]aminomethane hydrochloride)	Sigma Chemicals	T-3253
Valproic Acid (2-propylpentanoic acid) (sodium salt)	Sigma Chemicals	P-4543

Platelet Calcium Assay

Platelet Isolation

1. Draw 50 mL venous blood using a 19G butterfly needle attached into a 60 mL syringe containing 6 mL 3.15% sodium citrate (1:9). Detach and recap syringe, gently invert 3x and transport to laboratory
2. Transfer into a 50 mL polypropylene tube
3. Add prostacyclin (PGI_2) 0.06 µg/mL to each (3 µL stock in 50 mL blood), swirl
4. Centrifuge at room temperature (RT) 1310 rpm (375 g) 20 minutes
5. Transfer platelet-rich plasma (PRP) into 50 mL tube
6. Add PGI_2 0.3 µg/mL
7. Centrifuge RT 3200 rpm (2240 g) 10 minutes
8. Discard supernatant
9. Re-suspend pellet in 6.3 mL warm HEPES + 700 uL Citrate

Dye Incubation

10. Count and dilute platelets to 3.5 x 10^8
11. Invert X3, put parafilm on lid, incubate 37°C 30 minutes in dark

Platelet Re-Suspension

12. Add PGI_2 0.3 µg/mL
13. Centrifuge at room temperature 3200 rpm (2240 g) 10 minutes

14. Discard supernatant

15. Add 1000 uL HEPES and Remove

16. Repeat 3X total

17. Re-suspend in 7 mL HEPES

18. Dilute platelets to 1.0×10^8 cells/mL

Measurements

19. Incubate with drugs at room temperature for 40 minutes, pre-warm at 37°C and measure following schedule for duplicate samples

20. Add either 0.024 U/mL thrombin or 10 μM 5-HT

21. End-calibrations – 20 μL SDS (10%), 25 μL EGTA (0.5 M), 30 μL $CaCl_2$ (0.5 M)

Sodium Valproate (fresh daily) (600 µM final concentration)

MW 166.2
Therapeutic blood levels 300 – 700 µM

Make 1.5 mL of 300 mM stock solution:
0.07479 g in 1.5 mL ddH_2O. Serial Dilutions:

[Final VPA for 20 µL into 2 mL sample] µM	µL 300 mM stock	µL ddH_2O	
600	200	800	(60 mM)

Add 20 uL per 2 mL sample for final concentration of 600 µM

Lithium Chloride (fresh daily) (1.0 µM final concentration)

MW 42.39
Therapeutic blood levels 0.6 – 1.2 mM

Make 1.5 mL 1 M stock solution:
0.063585 g LiCl in 1.5 mL ddH_2O. Serial Dilutions:

[Final LiCl for 20 µL into 2 mL sample] µM	µL 1.0 M stock	µL ddH_2O	
1.0	100	900	(100 µM)

Carbamazepine (fresh daily) (40 µM final concentration)

MW 236.3
Therapeutic blood levels 20 – 50 µM

Make 1.5 mL 15 mM stock solution:
0.005317 g CBZ in 1.5 mL methanol. Serial Dilutions:

[Final CBZ for 20 µL into 2 mL sample] µM	µL 15 mM stock	µL ddH_2O	
40	267	733	(4.0 mM)

Amphetamine Sulphate (store –20°C) 1 mg/mL (10 µL) (50 ng/mL final conc.)
Peak plasma levels at 3 hours ~50 ng/mL

Dilute stock to 5000 ng/mL:

5 µL stock into 995 ddH_2O

Add 20 µL to 2 mL sample for final concentration 50 ng/mL

Thrombin Solutions (fresh weekly)

Dissolve 10 Units (T9010: lot# 85H9301) in 715 uL ddH_2O, Dilute to:

2.4 U/mL (0.024 U/mL final) 120 µL into 880 µL ddH2O

Add 20 µL to 2 mL samples

5-HT (store at –20°C)
MW 387.4

Make 10 mL of 1 mM stock 5-HT by dissolving 0.003874 g in 10 mL ddH_20. Add 20 µL to 2 mL (final concentration 10 µM)

Pluronic F-127 10 % (fresh daily)

Add 0.01 g into 100 µL DMSO

Fura-2 Solutions (fresh daily)

1. Dissolve 1 µg Fura-2AM per 1 µL DMSO (1 mM) (ie 50 µg in 50 µL DMSO)

PGI_2

Add 1 mL TRIS (above solution) into 1 mg PGI_2
Store in aliquots of 30 µL at – 80°C

Calibration Reagents

	[Soln]	[Final]	In 1 Litre	In 10 mL
EGTA (salt)	0.5M	6.25 mM	190.2 g	1.902 g
SDS	10 %	-	100 g	1 g

- Combine 500 μL each into microfuge tubes, freeze – 20°C

	[Soln]	[Final]	In 1 Litre	In 10 mL
$CaCl_2*2H_20$	0.5 M	7.5 mM	73.515 g	0.7653 g

- Aliquot 1000 μL into microfuge tubes, freeze – 20°C

HEPES Buffer (mM) (pH 7.3)

	mM	In 1 L
HEPES	10	2.383 g
NaCl	137	8.0 g
$CaCl_2*2H_20$	1.8	0.265 g
$MgCl2*6H_20$	1.0	0.214 g
Glucose	5.5	1.0 g
KCl	2.7	0.2 g
Na_2HPO_4 (dibasic)	0.4	0.05 g

- pH with 1.0 M Tris
- aliquot 45 mL into 50 mL polypropylene tubes, store –20°C

Tris Buffer 1.0 M, pH 9

	M	In 500 mL	In 25 mL
Trizma base	0.67	40.3 g	2.015 g
TRIS HCl	0.33	26.4 g	1.32 g

Sodium Citrate 3.15 %

	In 500 mL ddH_2O
Sodium citrate	15.75 g

aliquot 6 mL into 50 mL polypropylene tubes, store –20°C (i.e. 6 mL per 50 mL whole blood)